THE DETERMINATION OF ZINC IN PLANT MATERIALS

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Most of the methods proposed for the determination of small amounts of zinc are either laborious or inaccurate. Those put forward by Sylvester and Hughes (1936), Fischer and Leopoldi (1937), Hibbard (1937), Lott (1938), Westerhoff (1938), Schwaibold and Lesmüller (1939), Allport and Moon (1939), either involve too many manipulations or require an inconveniently large sample. More promising are those of Holland and Ritchie (1939) and of Cowling and Miller (1941), in which zinc is determined colorimetrically after it has been separated from other metals by an extraction with dithizone and carbamate. It is evident, however, that those who have tested the methods in collaborative work in the U.S.A. have had difficulties, particularly in obtaining solution of all of the zinc from the plant ash, and also because of contamination by zinc dissolved from the glassware. Both of these difficulties are discussed later.

Rogers and Gall (1937) have developed a spectrographic method, but special, rather expensive photographic plates are required. Stout, Levy, and Williams (1938) have given a brief outline of a method for determining zinc polarographically, after separating it and other heavy metals from the sample by dithizone. Their method was presumably based upon that of Heller, Kuhla, and Machek (1937), who seem to have been the first to realize the advantages of combining the dithizone and polarographic procedures. A somewhat similar method, which was also developed from the work of the three latter investigators, has been used with repeated modifications in this laboratory for the determination of zinc in fertilizers and plant materials over the past four years. Prior to this, attempts had been made to polarize a solution of the plant ash directly, in much the same way as has been suggested by Reed and Cummings (1940), but it was found that the procedure could only be applied satisfactorily to plants with a high zinc content.

OUTLINE OF PROPOSED METHOD.

The ground sample (1 gm.) is digested with a mixture of nitric, perchloric, and sulphuric acids, and the digest diluted, and then made alkaline with an ammonium citrate buffer containing dithizone. Zinc and other heavy metals are extracted (without removal of suspended silica) by means of chloroform, and the extract evaporated. Excess dithizone is destroyed by means of a further wet digestion, and the digest is taken to dryness. The residue is dissolved in 1 ml. of a mixture of ammonium chloride and potassium thiocyanate, and polarized between the potentials of 0·8 and 1·2 volts at the dropping mercury electrode.

APPARATUS.

In the course of one determination 4 different vessels are required. (1) A 50 ml. kjeldahl flask, preferably flat-bottomed. (2) A 100 ml. separating funnel, preferably the Squibb type with a short stem. No lubricant is used on the stopcock. (3) A small vessel for collecting the chloroform extracts. A straight-sided weighing bottle, diameter 30 mm., height 45 mm., volume 30–35 ml., with a ground-glass stopper has proved most convenient. It is sufficiently wide-mouthed for the evaporation of the chloroform extract and of the digesting acids, and also serves to contain the final 1 ml. of solution before it is transferred to the polarizing cell. (4) The electrolysis or polarizing cell. This is one of a type described by Heyrovsky (1936). An illustration of it and

of the accompanying gas-train has been given previously (Walkley, 1938). The narrowest part of the cell has an internal diameter of 8 mm., and when it contains 0·4 ml. of solution, the depth of this liquid is about 8 mm., which is quite sufficient for adequate immersion of the cathode. It is advisable to have 2 gas trains and 4 or 6 electrolysis vessels for routine work.

The cathode used is a length of capillary of fine and uniform bore (about 50μ) which does not need to be further reduced by drawing out in a flame. It is connected to the mercury reservoir by black, "surgical" rubber tubing. Many workers advocate glass for this junction, but it has proved to be inconvenient and unnecessary here. One such rubber tube has given 4 years service without affecting the working properties of the cathodes.

The polarograph is a Nejedly, model 8, photographic recording type, to which a current-compensator has been permanently fitted. It is kept in a room thermostatically controlled at approximately 20° C.

The errors involved when glassware containing zinc is used are shown in Table 1. For this test, vessels of two types of resistance glass were taken, one containing zinc and the other "zinc-free". After the vessels had been rinsed with acid and water, 20 ml. portions of various re-distilled reagents were evaporated to dryness in them. The small residue freed was then dissolved in 1 ml. of electrolyte and polarized as usual.

TABLE 1.

Zinc extracted from glassware by evaporation of 20 ml. of various reagents.

	Zinc extracted in micrograms.	
Reagent.	Woods resistance glass.	Pyrex glass.
Nitric acid 16 N	12·3	$< 0·1$
Hydrochloric acid 10 N	5·3	$< 0·1$
Water	2·4	$< 0·1$
Ammonia 3 N	0·9	$< 0·1$

Pyrex glass is used throughout and has proved very satisfactory. Strong redistilled acids have been stored in it for over 3 years without appreciable contamination by zinc. Alkaline solutions, however, increase appreciably in zinc content with long standing even in Pyrex glass, so that for the best results it is advisable to purify them just before use. Glassware should be washed with dilute acid to remove any adsorbed zinc, and finally rinsed with glass-distilled water.

A separate dip-pipette, kept covered in a beaker, is used for each reagent. All brass fittings which are handled, such as taps, are painted. Small removable fork-shaped supports, made from glass rod, are fixed horizontally to the stand supporting the separating funnels. These hold the stoppers when not in the funnels. The digestions of the plant material and of the dithizone, and also the evaporation of the chloroform, are conveniently carried out over Gilmer heaters (electric open-radiation type). Some transformer or other control of these is advisable. At full heat they consume about 600 watts.

REAGENTS.

Water: Redistil from glass vessels, discarding the first runnings.

Nitric acid (S.G. 1·42), Perchloric acid (S.G. 1·54), Sulphuric acid (S.G. 1·84): In each case distil acids of the above strengths from a retort or some other all-glass apparatus.

Ammonium citrate buffer: Take 5 gm. citric acid, 50 ml. ordinary distilled water, 200 ml. 4·0 M ammonia, and shake for 1 minute in a separating funnel with 10 ml. chloroform and 100 mg. powdered dithizone. Discard the chloroform and shake twice more with 10 ml. lots of chloroform only. The solution has a pH value of 10·7 and is orange due to dissolved dithizone. To neutralize the finished digest 25 ml. of this solution are used. Since dithizone oxidizes fairly rapidly in aqueous solutions, it is advisable to keep a stock mixture of citric acid and ammonia, and to add the dithizone to it on the same day as the plant extractions are to be carried out.

Chloroform: Redistil the commercial-grade reagent and add 1 p.c. of its volume of absolute alcohol.

Basal solution of 0·1 M ammonium chloride, 0·02 M potassium thiocyanate, 0·0002 p.c. methyl red: Dissolve 5·35 gm. ammonium chloride and 1·94 gm. potassium thiocyanate in water, add 5 ml. of 0·04 p.c. aqueous solution of methyl red and bulk to 1 litre. The best reagent grades of these salts may be used without purification.

Standard zinc sulphate: Make a 0·01 M solution of zinc sulphate and check by precipitation of the zinc ammonium phosphate. From an aliquot prepare a 0·001 M solution in a basal solution of the same composition as that stated above.

Mercury: The best reagent grades of mercury, after being shaken with distilled nitric acid and washed with water and dried, have been found satisfactory for the anode pool. Residues from the electrolysis cell can also be used for the anode pool after a similar treatment. For the cathode mercury it has been customary to purify by distillation at reduced pressure after the acid treatment.

Hydrogen: Commercial grades which have been prepared electrolytically seem to have a satisfactory low oxygen content.

For determinations on many fertilizer materials and on some plants of high zinc content the best reagent-grade chemicals can be used without any purification at all. The list given in Table 2 shows average values for the quantities of zinc introduced by the individual reagents when used in amounts necessary for the determination of zinc in 1 gm. of plant material.

TABLE 2.

Quantity of zinc (in micrograms) in reagents before purification.

Nitric acid	12·5 ml. contain	0·6
Perchloric acid	1·5 ml.	1·5
Sulphuric acid	1 ml.	0·1
Distilled water	30 ml.	0·9
Citric acid	0·5 gm.	0·6
Ammonia	20 ml.	0·5
Chloroform	15 ml.	0·0
		4·2

After purification this blank of 4·2γ can be reduced to less than 0·1γ, which may be neglected for most plant samples.

METHODS OF ASHING.

In all the methods listed in the introduction the sample is ashed in the dry way at a temperature of about 500° C., and the ash is dissolved as completely as possible in some acid, usually hydrochloric. In some cases any ash which is insoluble is filtered off; in others it is neglected. For materials low in insoluble-ash the dry-ashing method gives reliable results, but in this laboratory it has been found unsatisfactory for general work, and particularly for the analysis of cereal straws, which have a high silica content and a low zinc content. In such samples the ash seems to fuse slightly, so that some zinc is retained and not dissolved when hydrochloric acid is added. It is also very difficult to get a carbon-free ash in these cases. If the silica is removed by hydrofluoric and sulphuric acids, much of the zinc is released, but some still seems held up with the carbon, which is again difficult to remove. The magnitude of the errors involved is shown in Table 3.

TABLE 3.

The effect of removing silica and carbon from oat straw samples on the amount of zinc found.

Zinc contents in micrograms per gm. of dry matter.

	Silica not removed (dry ashing).	Silica removed, some carbon left (dry ashing).	Silica and carbon removed (wet ashing).
Oat straw A	6·9	10·4	11·3
Oat straw C	8·1	9·6	11·4

That retention of small amounts of other metals in the insoluble-ash may occur, has been pointed out by Clifford (1937) in the case of lead, and by Piper (1939) in the case of copper and manganese. Rogers and Gall (1937) showed that retention could also occur in the case of zinc but their work seems generally to have been overlooked.

It is usually recognized that the wet-ashing process is much more convenient, but several objections are often raised to it. These are: 1. The large amounts of acid and alkali required result in a high blank. 2. There is loss of zinc by volatilization. 3. The danger of explosions with perchloric acid. 4. The high price of good-quality perchloric acid.

The simple methods of purification used here dispose of the first objection, and satisfactory recovery tests, to be quoted later, dispose of the second. Though some risk may always be run whenever perchloric acid is handled, it can only be stated that over 600 zinc determinations by the wet-ashing process have been carried out without mishap. In addition to these, colleagues have successfully used the same reagents, though in somewhat different proportions, for still larger numbers of other digestions (Shapter, 1937; Piper, 1939). The purification of perchloric acid by simple distillation in an all-glass apparatus is considered a safe procedure by Hillebrand and Lundell (1929) and Kahane (1934). The main objection to direct distillation appears to be that some loss occurs through decomposition into chlorine and other substances. These losses do not occur when the reagent is distilled under reduced pressure, but the procedure is said to be complicated by a pronounced tendency to superheating (Smith and Goehler, 1931)

If sulphuric acid is omitted, the digestion is considered by Kahane and others to be unsafe. To obviate the chance of forgetting to add it, and for greater convenience, the acids may be mixed previously in the proportion of 10 ml. nitric, 1 ml. perchloric, and 1 ml. sulphuric.

With most plant materials a vigorous action accompanied by dense red fumes sets in immediately on warming, and in some cases a very persistent frothing may result. A drop of kerosene, if added at the outset, prevents this so completely that it is possible to put the flask on the heater and never to touch it again until digestion is complete.

When most of the nitric acid has gone, fumes of perchloric acid begin to appear, and soon a second vigorous action sets in, usually with some blackening of the digesting mass. The onset of this stage of the oxidation is fairly sudden but after a minute or two the action dies down equally quickly, leaving a colourless digest. The heating should be so adjusted from the outset that this stage is reached in about 30 minutes. With materials rich in protein or fats, the charring is more persistent, but all substances have so far yielded to the treatment except a sample of linseed with 40 p.c. ether-soluble fraction, which remained in the charred state and had to be digested twice. After a further 5 or 10 minutes heating the perchloric acid fumes become less dense, and sulphuric acid begins to reflux. The temperature should now be raised to the highest attainable on the Gilmer heater until the refluxing occurs at the base of the neck of the flask or higher. This is continued for 5 minutes, during which time any silica present is thoroughly dehydrated. If heating is continued much longer, bumping is apt to occur. Calcium sulphate, which is often present, may at times cause bumping during the early stages of digestion; during the later stages, even when present in large amount, it seems to promote boiling. For blank determinations, or for plant materials low in insoluble-ash, it is advisable to add a few pyrex glass beads. The finished digest, which consists chiefly of the 1 ml. of sulphuric originally added, is yellow when hot, but becomes colourless on cooling. When much manganese is present it is pink. The whole digestion is easily completed in 45–50 minutes.

The high price of perchloric acid prompted trials with sodium perchlorate, which is cheap, and which can be purified more easily. One ml. of 60 p.c. perchloric acid is approximately equivalent to 1 gm. of sodium perchlorate. This may be dissolved in 1 ml. of water, but the specific gravity of the solution is so close to that of chloroform that purification becomes difficult. A 50 p.c. solution of the salt can, however, be purified easily, and the following alternative procedure can be recommended: Take 50 gm. of sodium perchlorate and bulk to 100 ml. with water. Add about 5 drops of 0·1 M caustic soda till the reaction is alkaline to phenol red or thymol blue. Filter if necessary, and then remove any heavy metals by extraction with dithizone in chloroform. For the digestion 2 ml. of this solution are used in place of the 1 ml. of perchloric acid. As with the latter, the sodium perchlorate solution can be mixed with nitric and sulphuric acids in the desired proportions. The course of the digestion is exactly the same as before, though the final temperature reached is somewhat higher (360° C. as against 330° C.). On cooling, the digest solidifies, but easily dissolves in the 15 ml. of cold water which are always added before neutralization.

NEUTRALIZATION OF DIGEST AND EXTRACTION OF ZINC.

If the dithizone extraction is carried out at about pH 8 without filtering off the silica, low values for the zinc content are obtained. That this zinc is retained by the silica can be shown by filtering off the silica after extraction, removing it by sodium fluoride treatment, and determining zinc in the solution so obtained. If, on the other hand, a sufficiently large excess of ammonia is added to give a pH value of about 10, there appears to be very little retention. The effect of the increased alkalinity on the partition of zinc has been described elsewhere (Walkley, 1942). The amount of zinc extracted at this high pH value in the presence of silica has frequently been compared with that extracted after removal of the silica by sodium fluoride treatment. From Table 4 it will be seen that the differences between the two procedures are small.

TABLE 4.

Comparison of zinc contents determined after wet digestion, and extraction either in the presence or absence of silica.

		Silica content (p.c. dry matter).	Zinc content in micrograms per gram dry matter. Silica removed.	Silica present.
Oat, straw	A	2·7	11·3	11·3
Oat, straw	B	2·2	11·4	11·1
Wheat, whole plant	A	2·8	12·8	13·0
Wheat, whole plant	B	2·8	13·3	13·3
Ryegrass, leaf and stem		5·6	45·0	45·2
Wild geranium		1·2	150	152

The sodium fluoride procedure nevertheless may be useful in the event of plants being encountered with higher ratios of silica to zinc than are mentioned in this paper. To remove the silica by this method, complete the digestion as usual, add 15 ml. water, bring to the boil, and transfer to a platinum basin. Add 5 ml. of a 5 p.c. solution of sodium fluoride (which has been previously purified by dithizone extraction) to the Kjeldahl flask, and warm for a few seconds.

The film of silica on the flask quickly dissolves. Pour into the dish, and rinse the flask into the dish with water several times. No observable attack on pyrex glass occurs. Evaporate the digest until the sulphuric acid begins to fume. Cool and add ammonium citrate and ammonia as usual. Hydrofluoric acid is not used as it is so difficult to purify. Ammonium fluoride has been found less suitable than the sodium salt, because it tends to spurt when decomposing.

The only other relatively insoluble material likely to be found is calcium sulphate. This also adsorbs zinc, but has not given trouble because in all cases so far it has dissolved in the 50 ml. of aqueous solution. When large amounts of calcium sulphate are present, the addition of sodium hexmetaphosphate is useful in retaining it in solution. The diluted digest containing the calcium sulphate and sulphuric acid must be carefully neutralized with ammonium citrate and ammonia before boiling it with the metaphosphate, because the latter would otherwise revert to the orthophosphate. After standing some time a clear solution is obtained; whereupon the remainder of the ammonia is added, and the zinc extracted as usual. Ten ml. of 10 p.c. sodium hexmetaphosphate are sufficient to bring 0·3 gm. calcium sulphate into solution. Larger amounts of metaphosphate tend to reduce the efficiency of the zinc extraction, presumably through formation of sodium-zinc-metaphosphate complexes. This procedure has been used successfully in the analyses of some fertilizer samples (Walkley, 1940).

Upon neutralizing a digested sample with the 25 ml. of ammonia buffer containing dissolved dithizone, the mixture becomes cloudy, due to zinc dithizonate in suspension. This does not show any tendency to settle out, and so is easily transferred quantitatively to the separating funnel.

If carbon tetrachloride is used in place of chloroform, the efficiency of extraction is considerably reduced.

Removal of Dithizone.

It is not easy to destroy dithizone completely at a low temperature either by dry or wet ashing. Other workers have recommended digesting with bromine and hydrochloric acid, nitric acid, aqua regia, or a mixture of perchloric acid and perhydrol, but none of these reagents were found to be satisfactory for the relatively large amounts of dithizone used here. Various forms of the nitric-perchloric-sulphuric acid digestion gave the best results. If the dithizone can be boiled with the digesting mixture then only a few drops of each acid are necessary; but since this is not possible without loss of contents if the operation is to be done in the small 30 ml. bottle previously mentioned, a larger amount of perchloric acid is required. A mixture of 2·5 ml. nitric acid, 0·5 ml. perchloric acid and 2 drops of sulphuric acid, if evaporated at a temperature just below its boiling point will oxidize completely all the dithizone used here. It is important to heat the mixture as strongly as possible without actually bringing it to the boil. In order to remove the sulphuric acid without overheating it is advisable to put the bottles in a muffle kept between 300 and 350° C. for 2 or 3 minutes. A perfectly dry carbon-free ash is left.

Sodium perchlorate has never been used for this oxidation, as it would leave a large residue which would probably interfere with the polarographic determination.

THE POLAROGRAPHIC DETERMINATION.

The ideal basal solution should have a pH value greater than 7, because dissolved oxygen can be removed from it by the simple addition of a sulphite. A gas-train then becomes unnecessary, and a simpler type of polarizing cell can be used. Unfortunately in all of the alkaline solutions so far tried, the zinc and cobalt steps coalesce. This applies to acetates, oxalates, and thiocyanates, in the presence of excess ammonia; and also to solutions of ammonia alone, or to mixtures of ammonia and ammonium chloride. If, as seems probable however, the cobalt contents of plants are usually small compared with their zinc contents, no great errors might result in determining the sum of the zinc and cobalt. This can be done by using a basal solution of ammonium chloride and ammonia, containing a sulphite and gelatin. A similar solution was suggested by Heyrovsky (1939) for the determination of copper, cadmium, and zinc in zinc blendes. With the proviso just mentioned in regard to cobalt, it would seem that a determination of both copper and zinc (plus cobalt), on the one plant sample and on the one polarogram, is feasible. There does not appear to be any basal solution devised so far, which permits of a simultaneous determination of copper and zinc, and which at the same time enables zinc, nickel, and cobalt to be resolved. Until more information is available on the relative concentrations of zinc, nickel, and cobalt in plants, it has been considered advisable to retain the ammonium chloride-potassium thiocyanate mixture, which, as repeated experience has shown, gives a good resolution of the above three metals.

Stout, Levy, and Williams (1938) have used a similar solution for the same purpose. It has a pH value of 5·2, so that the addition of sulphite to remove oxygen is not permissible. Either methyl red or gelatin are useful maximum suppressors for this particular solution, but the former is preferred as it keeps better. It has been shown elsewhere (Walkley, 1941) that in the above solution the height of the zinc step is linearly related (to within ± 1·5 p.c.) to the concentration of zinc, over the range 3 to 300γ per ml. This corresponds to a range of 3 to 300γ per gm. of dry matter, within which range all the plant samples so far encountered have been found to lie. Below 3γ per ml. it is advisable to use a calibration curve. It will be seen from curves 4–6, Fig. 1,

that even with less than 1γ of zinc, the steps are well defined and measurable by the method indicated.

The capillary is calibrated by taking several measurements of the step-height of the standard 0·001 M zinc solution at approximately 20° C., and adding or subtracting 1·7 p.c. for each degree centigrade below or above 20° C. The step-height of the polarogram of the unknown zinc solution is corrected in the same way, and its zinc concentration then calculated by simple proportion.

There are several other metals in plants besides zinc which are extracted by dithizone, and which give increases in current on the polarogram. These are copper, lead, cadmium, nickel, and perhaps cobalt. The applied potentials corresponding to the points of inflexion of their steps are (approximately) 0·0, 0·4, 0·6, 0·7, and 1·3 volts. Experience has shown that the last four metals are usually far less abundant than zinc and published analyses indicate that copper is also usually less abundant. No analyses have been seen in which the copper content exceeded twice the zinc content. Bearing these restrictions in mind, the effect of the presence of the metals on the height of the zinc step was investigated, the concentration of added metal being one-tenth of the concentration of the zinc in the case of nickel, cobalt, cadmium, and lead; and twice the concentration of zinc in the case of copper. All these additions were without influence on the height of the zinc step. When the copper concentration was made 10 times as great as the zinc concentration, the height of the zinc step was increased by 5 p.c.

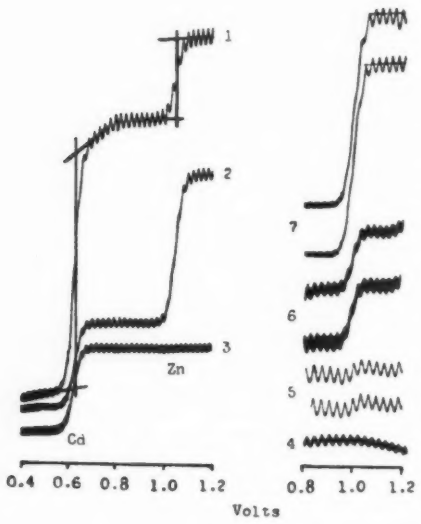

Fig. 1. Polarograms obtained in the course of zinc determinations.

	Galvo sensitivity.
Curve 1. Oat Sample containing 5·0 micrograms of zinc per gm.	1/10 of maximum.
Curve 2. Oat Sample containing 28·4 micrograms of zinc per gm.	1/30 of maximum.
Curve 3. Blank	1/30 of maximum.
(Curves 1–3 illustrate internal-standard method.)	
Curve 4. Blank (Basal solution alone)	Maximum.
Curve 5. Blank showing less than 0·1 microgram of zinc (all reagents purified)	Maximum.
Curve 6. Blank showing 0·4 micrograms of zinc due to impure perchloric acid	Maximum.
Curve 7. Wild geranium sample containing 152 micrograms zinc per gm.	1/100 of maximum.

Substances not extracted by dithizone may find their way into the chloroform, either by faulty manipulation of the stopcock of the separating funnel, or merely by solution of some of the aqueous liquid in the chloroform. Substances of this type which may possibly be expected to interfere with the zinc determinations are iron and phosphorus. Once again, however, there was no appreciable effect due to the presence of a 20-fold excess of phosphorus as potassium phosphate; or of a 2-fold excess of iron as ferric sulphate. With substances relatively rich in copper the solution in the polarizing cell may become cloudy, due probably to the formation of some cuprous thiocyanate. This does not interfere with the zinc determination.

The Method of Reed and Cummings.

If the dithizone extraction is omitted, the final solution to be polarized has to be much more dilute, owing to the limiting solubility of the major constituents of the ash. In the method of Reed and Cummings (1940), for example, the zinc contained in 2 gm. plant material is finally dissolved in 25 ml. of basal solution, whereas in the method presented here the concentration of zinc in the basal solution is the same as it is in the plant material. For substances of low zinc content (10 or less γ per gm.) the concentration of zinc in the final solution of Reed and Cummings is less than one γ per ml., which is approaching the range at which the accuracy of the polarographic method begins to fall off. This and similar procedures have been found unsatisfactory unless the sample contains about 50 or more micrograms zinc per gm. With siliceous ashes of low zinc content very low results have been obtained.

The Use of Cadmium as an Internal Standard.

The internal standard method of determining metal concentrations polarographically, as suggested by Forche (1938), has been used by Goode and Hall (1940), and by Cholak and Bambach (1941) for the determination of lead. The former used thallium as the standard of reference, and the latter cadmium.

Cadmium seemed the most useful standard for the determination of zinc in plants because polarographic analyses had repeatedly failed to reveal it in amounts which would lead to any disturbance. The same is true of thallium but the thallium and lead steps overlap, and lead is often found in amount sufficient to increase the height of the thallium step. There is also a certain amount of overlapping of the cadmium and nickel steps, which may introduce a small error at times in plants with a high ratio of nickel to zinc. The general advantages claimed for internal-standard methods over direct ones are that the measurements obtained are independent of the viscosity of the solution, the height of the reservoir, the characteristics of the capillary, and the temperature. In this particular instance, it is only the temperature variation which is of importance, and if no temperature control is available, the internal-standard method is probably the better. It can be used over a very large temperature range. For example, the ratios of the heights of the zinc and cadmium steps for a plant sample determined at 10° C., 20° C., and 35° C. proved to be so close that the calculated zinc contents worked out at 28·8, 29·1, and 29·3γ per gm.

Some curves obtained are shown in Fig. 1, curves 1–3. As before, a 1 gm. plant sample was taken, and the metals extracted by dithizone were dissolved in 1 ml. of basal solution of the same composition as usual, but containing cadmium sulphate of concentration 0·00025 M. In sample 2 the cadmium step is well marked, whereas in sample 1 the curve continues to rise even after an applied potential of 0·7 volts has been reached. If cadmium is not added, a step is still obtained at this potential, which corresponds to the deposition potential of nickel. Sample 1 has the highest ratio of nickel to zinc so far encountered, but even in this case very little error is made if the cadmium step is measured in the usual way as shown. Comparisons of the direct and internal-standard methods have been made on 50 different plants comprising 24 different species. In Table 5 are shown some of the results, including those for which the differences between the two methods are greatest.

TABLE 5.

Comparison of direct and internal-standard methods.

Zinc contents in micrograms per gm.

	Direct method.	Internal-standard method.	Difference.	Percentage difference.
Oat sample 1 (see Fig. 1)	4·9	5·0	0·1	2·0
Oat sample 2 (see Fig. 1)	28·0	28·4	0·4	1·4
Rye	21·4	20·8	— 0·6	— 3·5
Tick bean	75·6	73·0	— 2·6	— 3·6
Snail medic	28·4	29·4	1·0	3·4
Phalaris tuberosa	25·7	26·7	1·0	3·7

Mean percentage difference (6 plants) ± 2·9.
Mean percentage difference (50 plants) ± 1·6.

Though more evidence is desirable, it seems reasonable to assume that the naturally occurring cadmium and nickel in plants is probably not sufficient to cause serious errors when cadmium is used as a standard for zinc determinations.

PROCEDURE.

Take approximately 1 gm. of ground plant material in a weighing bottle, and dry overnight at 105° C. Weigh and transfer to a Kjeldahl flask. Add 10 ml. nitric acid, 1 ml. perchloric acid (or 2 ml. sodium perchlorate solution), 1 ml. sulphuric acid, and a drop of kerosene. Heat until all the nitric and perchloric acids have been driven off, and the sulphuric acid has been refluxing for about 5 minutes. Cool. Add 15 ml. water and bring to the boil over a bunsen flame, keeping the flask agitated to prevent bumping. Allow to cool and add 25 ml. of ammonia buffer containing dithizone.

To the separating funnel add approximately 5 ml. of chloroform and then the neutralized digest and rinsings. Shake vigorously for about 1 minute and allow to stand. Run off as much of the chloroform layer as is possible without allowing

any of the aqueous layer, or the silica which collects at the interface, to enter the stopcock. Repeat with a second and third 5 ml. portion of chloroform. There is no need to shake the third portion for one minute. It merely serves to rinse out the separating funnel and its stopcock. Collect all three portions of chloroform in the small straight-sided bottle, and evaporate to dryness. Add 2·5 ml. nitric acid, 0·5 ml. perchloric acid, and 2 drops of sulphuric acid, and again heat, keeping the temperature as high as possible, but avoid boiling. When the sulphuric acid is fuming strongly, transfer to a muffle at 300–350° C. for 2–3 minutes. To the cool, dry residue add 1 ml. of the mixed basal solution, stopper the bottle, and let stand a few minutes for the zinc to dissolve.

To the dry polarizing cell add sufficient mercury to form the anode pool (about 0·3 ml.), and then pipette in approximately 0·4 ml. of the solution containing the zinc. Put the capillary into position and pass hydrogen for 5 minutes. Turn off the hydrogen, note the temperature near the electrode, and apply a polarizing potential of 0·8 volts. Record the current-voltage curve over the range 0·8-1·2 volts. Correct the step-height observed for the temperature variation, and calculate the concentration of zinc in the final solution as explained earlier.

RECOVERY TESTS ON PLANT MATERIALS AND ACCURACY OF THE METHOD.

The percentage recoveries of zinc and of cadmium when added in both blank and plant determinations are shown in Table 6. In all cases the zinc and cadmium were added in solution to a Kjeldahl flask, and evaporated to dryness before the plant sample was added. The method as stated does not claim to be suitable for the determination of cadmium in plants, since the amounts present are so small. A determination may be possible if a much larger sample is taken. It is clear, however, that even as little as 1 microgram, if present, would easily be detected in the method as it stands.

TABLE 6.

Recovery of zinc and cadmium added in plant and blank determinations.

	Metal present in reagents or plant or both.	Metal added.	a Total metal present.	b Metal found.	$\frac{100b}{a}$
		Cadmium.			
		Amount of metal in micrograms.			
Blank	0·15*	1·12	1·27	1·12	88
Blank	0·15*	1·12	1·27	1·31	103
Wheat flour, 1 gm.	0·15*	1·12	1·27	1·17	92
		Zinc.			
Blank	0·73*	1·64	2·37	2·46	104
Blank	0·2	16·4	16·6	16·8	101
Blank	0·2	32·7	32·9	33·6	102
Blank	0·2	81·7	81·9	80·2	98
Blank	0·2	164	164	164	100
Blank	0·2	327	327	337	103
Wheat flour, 1 gm.	4·5	9·0	13·5	13·2	98
Prune leaves, 1 gm.	8·0	17·2	25·2	25·8	102
Oat, whole plant, 1 gm.	8·6	16·4	25·0	24·5	98
Vine leaves, 1 gm.	17·2	33·6	50·8	52·1	103
Wheat germ, 1 gm.	64·5	132	196	200	102
Soy-bean meal, 1 gm.	68·1	132	200	205	102

* Due to impure perchloric acid.

The figures in the last column indicate the degree of accuracy of the method. These are single determinations. When replicate determinations are carried out on the same sample, the results show a variability similar to that indicated in the last column.

The following advantages are claimed for the method. (a) The ashing procedure is very simple. (b) There are no significant disturbances due to insoluble ash. (c) The blank is easily reduced to a negligible amount. (d) The polarographic procedure used here is specific for zinc, but may also give information about other metals if present. (e) There is no need to know the approximate zinc content of the plant before deciding on the size of the sample, because the procedure is identical for amounts of zinc between 3 and 300 micrograms.

When analyses are done in series the average time for one determination, from the weighing of the oven-dry sample to the final computation, is about one hour.

SUMMARY.

A method is described for the determination of zinc on one-gram samples of plant material, using a wet ashing procedure, dithizone extraction and polarographic determination.

It is shown that sodium perchlorate can be used instead of perchloric acid for the wet ashing.

The errors introduced by impure reagents and certain types of glassware are discussed. By simple methods of purification the blank may be reduced to a negligible amount.

The dithizone extraction can be carried out in the presence of suspended silica.

Where temperature control is not possible, the internal-standard method may be used, cadmium serving as the standard of reference.

Recovery tests show that the precision and reproducibility of the method are of the order of $\pm$ 3 per cent.

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STUDIES OF THE FLAX PLANT

1. PHYSIOLOGY OF GROWTH, STEM ANATOMY AND FIBRE DEVELOPMENT IN FIBRE FLAX

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INTRODUCTION.

In the present work the primary objectives were to study the developmental anatomy of the fibre bundles of flax, and to determine the effect thereon of artificial drought applied from the time of commencement of flowering. In planning the experiment, however, it was thought desirable to include parallel studies of the physiology of the growth of the plant as a whole, and of limited aspects of carbohydrate and nitrogen metabolism. This plan provided closely interrelated sets of data which are of special interest, even though the anatomical section is almost entirely confined to a single structural element in the stem.

The treatment of the physiology of growth is based on earlier work from this laboratory (Ballard and Petrie, 1936; and Williams, 1936, 1939), and only outstanding effects of treatment, and specific features associated with the flax plant are considered in the discussion. The type of low water treatment was suggested by earlier experience with barley, rye-corn and tobacco; the data of the latter experiments, however, are as yet unpublished. As far as the author is aware, the anatomy of the fibre bundles has not previously been studied from a developmental point of view. Data relevant to carbohydrate and nitrogen metabolism are reserved for a later paper of this series, though the protein-nitrogen contents of the leaves are used below in the growth analysis.

SYMBOLS.

L Length of stem.
W Dry weight of a 10 cm. sample from the centre of the stem.
F_A Cross-sectional area of "true" fibre just below the 10 cm. sample.
F_W Weight of fibre per stem.
$F_{\%}$ Percentage weight of fibre.
R Relative growth rate.
E_W Net assimilation rate on a dry weight basis.
E_P Net assimilation rate on a protein-nitrogen basis.

EXPERIMENTAL PROCEDURE.

On May 6, 1941, seeds of flax (*Linum usitatissimum* L. variety Liral Crown) were planted in enamel pots containing 15 kg. of a thoroughly mixed soil preparation consisting of 2 parts of loam from the fields of the Waite Institute and one part of washed river sand. To the upper layer of soil in each pot, 3 gm. of superphosphate were added. During the filling of the pots, soil samples were taken for percentage moisture and moisture equivalent determinations. From the latter, the permanent wilting percentage was calculated on the assumption that the ratio of moisture equivalent to permanent wilting percentage was the same as that found by Marshall and Williams (1942) for a similar soil mixture. Sixty seeds were planted in each of 78 pots, and those which failed to germinate were replaced by seedlings so as to ensure an even spacing of the plants. The pots were randomized in the glasshouse within six blocks according to treatment and time of harvesting for which they were destined.

Harvests were made on June 18, July 9 and 30, August 12, September 1 and 19, October 14 and 31. After harvest 4, the soil moisture level of half of the then remaining pots was decreased

to simulate drought conditions. The pots receiving the higher moisture treatment were maintained approximately at the moisture equivalent (M.E. = 14·15 p.c. oven-dry soil) by watering at frequent intervals, whereas those receiving the low moisture treatment were allowed to fall to the permanent wilting point (4·56 p.c. oven-dry soil) before being brought back to the moisture equivalent. The fluctuations with time in soil-moisture content in relation to harvesting dates can be seen in Figs. 1 and 2. There were six replicates for each treatment-harvest class and, in spite of precautions taken, the final number of plants per pot varied as indicated in Table 1. The six remaining pots, three from each treatment, were harvested on October 14, and were experimentally retted by courtesy of W. L. Greenhill, Division of Forest Products, C.S.I.R.

At each harvest two stems were taken at random from each pot for anatomical study, and suitable samples of leaf and stem material were set aside for carbohydrate estimations. All plants were cut at ground level, and various parts separated as indicated below. The leaves were stripped from the stem, and, after flowering (August 19), the inflorescence was cut immediately below the first branch of the panicle. All inflorescence leaves were included in the leaf fraction. The root systems were obtained by sieving under water until practically free from soil; foreign matter which remained was subsequently separated by hand. The fresh weights of leaves and stems were determined immediately after separation, and all fractions were dried as quickly as possible in ovens at 85° C. under forced draught. At the final harvest, only stem and inflorescence weights were recorded.

The method adopted for preparing sections and for determining the fibre content of the stems was similar to that used by Schmalfuss (1936). The two stems taken for anatomical examination were measured for length (L), which was taken as the distance from the cotyledons to the top of the stem in the younger plants, but to the first inflorescence branch in older plants. A 10 cm. sample was cut from the middle of the stem and dried at 100° C. to constant weight (W). Immediately below the position of the sample, many hand sections were cut and placed in strong glycerine; two of these were selected and mounted in glycerine. When possible, all sectioning and mounting was completed on the day of harvest. Outline drawings of the stem and fibre bundles were made with the aid of a projection microscope. The cross-sectional area of the stem and the total area of fibre bundles were estimated with a planimeter. In order to find the percentage area represented by "true" fibre, a photomicrograph was taken of a more highly magnified portion of each section, and from this the areas occupied by fibre and their lumina were found. The difference between the two values gave the area of "true" fibre, and this was expressed as a percentage of the area of the fibre cells. This percentage was then used in finding the total area of "true" fibre (F_A) from the drawings made on the complete section.

Lignification of the fibre cells was observed in sections stained with phloroglucin. The number of fibre cells showing lignification in each section was counted and expressed as a percentage of the total number of fibre cells. The values thus obtained were used as indices of the amount of lignification occurring in the section.

CALCULATION OF DERIVED QUANTITIES.

From the primary data (Tables 1 and 2) the following derived quantities have been calculated.

Growth analysis. Relative growth rate (Ballard and Petrie, 1936); net assimilation rate on a dry weight basis (this quantity is identical with the unit leaf rate of Ballard and Petrie, *loc. cit.*); net assimilation rate on a protein basis (Williams, 1939). Leaf, stem, root and inflorescence weight ratios are the ratios of the dry weights of the parts concerned to the dry weight of the whole plant.

Fibre estimations. It may be assumed that the walls of the fibre cells are of pure cellulose (Anderson, 1927) with a specific gravity of 1·5. An estimate of the total weight of fibre (F_W) per single plant stem may be derived from the equation $F_W = F_A \times L \times 1{\cdot}5$. (1)

These values for F_W also involve the assumption that F_A is the true mean value over the entire length of the stem. Similarly the percentage weight of fibre ($F_{\%}$) in the 10 cm. sample may be estimated from the equation

$$F_{\%} = \frac{F_A \times 10 \times 1{\cdot}5}{W} \times 100. \quad (2)$$

In this connection the work of Jakovlev (1932) and Tammes[1] (1907) is of interest. Jakovlev divides the flax stem somewhat arbitrarily into three zones each having certain characteristics. These are: (a) the lowest quarter of the stem, where the development of secondary xylem is greatest, and the percentage of fibre is least; (b) a central zone where, in spite of marked differences in cross-sectional areas of both xylem and fibre bundles, the percentage fibre content is relatively uniform; and (c) the upper quarter, where the principal anatomical elements are least

[1] The original monograph was not available to the author, consequently the statement given here is derived from a paper by Robinson (1933).

developed and the fibre percentage is intermediate between those of the first two zones. It should be noted that the index of fibre percentage used by Jakovlev was the area of fibre bundles expressed as a percentage of the stem area less that of the pith. Tammes studied the distribution of fibre cells and bundles throughout the length of the stem. She found that, whereas the number of fibre bundles was relatively constant, the number of cells per bundle was greatest in the median portion of the stem.

In order to gain further information about the distribution of fibre within the flax stem, cross sections were made at regular intervals along a single stem. As no material from the main experiment was available, a stem grown under practically identical conditions and 95 cm. in length was selected for this purpose. The data are presented in Table 3 and graphically in Fig. 3. The variation throughout the length of the stem in the area of fibre and of other tissues will be discussed in detail in a later section of the paper. Here we are solely concerned with its bearing upon the validity of equations (1) and (2).

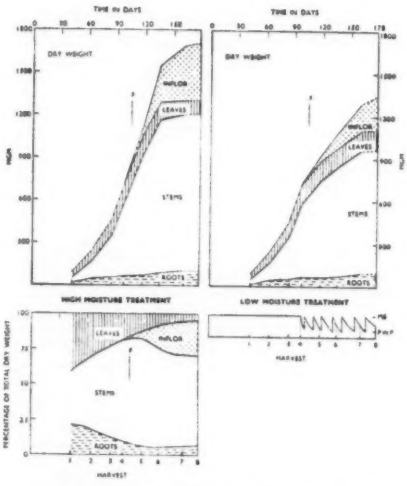

Fig. 1. The two upper Figs. show the dry weights of plant parts plotted additively, and illustrate the effects thereon of the soil-moisture treatments. Below, on the left, the relative proportions of the plant parts are shown for the high moisture treatment. Below, on the right, is a diagram representing the variation in soil-moisture content (between the moisture equivalent, M.E. and the permanent wilting percentage, P.W.P.) for pots of the low moisture series. The vertical lines marked F in this and succeeding Figs. indicate the time of commencement of flowering.

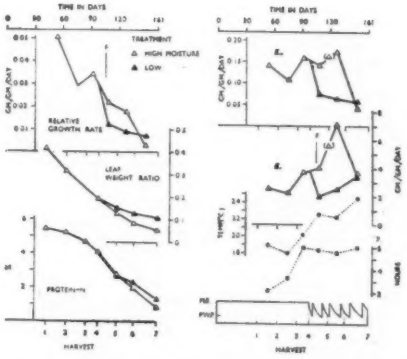

Fig. 2. Curves of relative growth rate, leaf weight ratio, leaf protein-nitrogen, and net assimilation rate on dry weight (E_W) and protein (E_P) bases. The climatic indices, mean maximum daily temperature and mean hours of bright sunshine per day for the appropriate inter-harvest periods are plotted above a soil moisture diagram (see Fig. 1).

Since fibre and xylem together make up the bulk of the solid matter of the stem (the remainder consisting chiefly of thin-walled parenchyma and of hollow pith), it is clear that an index of fibre percentage, based on the ratio of the area of fibre bundles to the sum of the areas of xylem and fibre, is better than that used by Jakovlev. The latter index is rendered less reliable by the fact that the relative proportion of parenchymatous tissue in the stem varies considerably throughout its length (see Fig. 3). The values of our index of fibre percentage increase rapidly from the base up to the position of the third cross section, they remain relatively constant for sections 3–7 inclusive, but the values for sections 8–10 are still greater. Values for this index calculated from Jakovlev's data show similar variations for comparable positions along the stem, though the increase in the upper portion of the stem is much less pronounced.

The data of Table 3 provide a means for estimating the magnitude of the errors involved in the assumptions made in the calculation of the derived quantities F_W and $F_{\%}$. In the mature

stem studied, F_4 overestimates the true mean value by 16 p.c., hence F_W is also in error. Without further investigation, it is impossible to say whether this error would be of the same order for immature stems, but this does not destroy the value of F_W as an index of fibre yield at a given stage of growth. Although $F_{\%}$ is not intended as a precise measure of the fibre percentage for the whole stem, but only for estimating the effects of time and treatment thereon, it is of interest to note that, for the mature stem studied, the mean value of the ten ratios is practically the same as that for the central 10 cm. section (i.e. the mean of sections 5 and 6). This implies that the sample used for the estimation of $F_{\%}$ was quite representative. An error, however, is introduced in the calculation of $F_{\%}$ according to equation (2) by assuming that F_4 is the true mean value for the 10 cm. strip. It should be remembered that F_4 is the area of "true" fibre at the base of the strip, and from the data of Table 3 it will be seen that $F_{\%}$ is overestimated by about 10 p.c.

GROWTH OF THE PLANT AND ITS PARTS.

The basic growth data of the experiment are presented in Table 1 and the dry weights of the various plant parts are plotted additively in the upper portion of Fig. 1. At the final harvest, only stem and inflorescence weights were taken, but, in order to complete the picture, it was assumed that the root and leaf weights remained as at harvest 7. The growth curve for the whole plant with high moisture treatment has the sigmoid shape typical of annual plants, but with the low moisture treatment the curve exhibits a marked discontinuity at harvest 4. Low moisture treatment commenced at this stage, which coincided approximately with the appearance of the inflorescence.

Effects of time and treatment (Fig. 1). As might be expected in a variety grown primarily for its stem yield, Liral Crown flax has a remarkably high stem weight ratio at maturity. In this respect it greatly exceeds that of the linseed variety Punjab (unpublished data), which in turn has a much higher inflorescence weight ratio. Liral Crown flax has very small leaf and root weight ratios at maturity, but this is not true for early stages of growth; thus at harvest 1 they have values of 0·416 and 0·211 respectively. It is also of interest to note that, as for tobacco (Petrie, Watson and Ward, 1939), the absolute weight of the root system increases continuously. In this respect both plants differ from wheat (Ballard and Petrie, 1936) and oats (Williams, (1936), from the roots of which appreciable quantities of dry matter are translocated during the development of the grain. These differences are attributable to the smaller inflorescence weight ratios of fibre flax and tobacco, and to the fact that secondary thickening occurs in the roots of both these plants. The data for Sudan grass (Ballard and Petrie, 1936) grown with adequate nitrogen supply support these suggestions in that the inflorescence weight ratio is exceptionally small.

TABLE 1

Dry weights in mg. per plant.

Harvest.	Plants per pot (range).	Leaves.	Stems.	Inflor.	Roots.	Total.
1	58–60	36·3	32·6	—	18·4	87·3
2	58–61	78·1	126·0	—	43·5	247·6
3	59–61	109·4	293·8	—	56·6	459·8
4	59–63	138·2	512·7	—	68·5	719·4
5H	58–60	146·1	842·2	57·5	71·5	1,117·3
6H	58–60	127·4	1,072·1	254·0	89·4	1,542·9
7H	57–60	99·3	1,096·6	380·0	104·8	1,680·7
8H	58–61	—	1,101·4	401·5	—	—
5L	59–61	143·8	678·2	34·1	64·2	920·3
6L	58–61	136·1	767·5	102·8	74·2	1,080·6
7L	58–60	142·4	864·0	188·7	94·4	1,289·5
8L	58–60	—	873·6	229·9	—	—

H = high moisture. L = low moisture.

The growth of the whole plant, as reflected in yield of dry matter, is greatly depressed at harvests 5 and 6 by the low moisture treatment, but, thereafter, the increments between harvests are slightly greater than with the high moisture treatment. It is evident that the depression in yield is sustained primarily by the stems and inflorescences, though the root weights are consistently

depressed at harvests 5–7 by more than 10 per cent. The absence of a significant depression in leaf weight is doubtless attributable to the fact that the maximum was almost attained at harvest 4. The fact that leaf dry weight decreases after harvest 5 with high moisture treatment, whilst it remains constant with low moisture treatment can probably be accounted for by the greater drain from the leaves by the larger inflorescence, and a consequent and unavoidably high loss due to the shedding of the older leaves. Comparing low with high moisture treatment, the percentage depression in dry weight is greater in the inflorescence than it is in the stem; this is to be expected, since the whole of the inflorescence was elaborated after the commencement of treatment. Many of the observed effects discussed above are expressions of delayed development in the plants receiving limited water supply. The uppermost leaves of the latter, for instance, remained green right up to the final harvest, whereas with high moisture treatment these were completely senescent.

Growth analysis (Fig. 2). As the first harvest of this experiment was made 43 days after sowing, the initial rapid rise in relative growth rate was not observed (see Williams, 1936, Exp. 2). From the first harvest interval, the curve exhibits a general downward trend in which, as will be seen below, fluctuations are mainly due to the changing environment and to experimental error. The effects of low moisture treatment on the yield of dry matter discussed above are reflected in a marked depression of R over harvest intervals 4–5 and 5–6, and in a reversal for harvest interval 6–7.

The relative growth rate may be analysed into two components, the leaf weight ratio and the net assimilation rate per unit weight of leaf. The latter quantity may be further analysed into the relative protein content of the leaves[2] and the net assimilation rate on a protein basis. There is a continuous decline in the leaf weight ratio throughout the period of the experiment, the decline being delayed by the low moisture treatment. It will be apparent that the time trend of R is largely determined by that of the leaf weight ratio. Relative protein content also decreases with time though this decrease is small until after harvest 3. At harvests 6 and 7 the relative protein content of the leaves is greater with low moisture than with high moisture treatment.

The two rate factors, E_W and E_P, are presented in Fig. 2 on the same time scale as the two available climatic indices, mean maximum temperature and mean hours of bright sunshine. A diagrammatic representation of the soil moisture treatment is also given. Williams (1939) has shown for wheat, oats and Sudan grass that E_P is determined primarily by factors of the environment, particularly temperature, whereas E_W tends to fall with time. The data for flax grown with the high moisture treatment confirm this finding, even though the trends in E_W and E_P do not differ greatly prior to flowering. This is clearly due to the small decrease, during this period, in the intrinsic factor (protein content) determining E_W. It seems probable that values of E_W and E_P for harvest intervals 4–5 and 5–6 may be low and high respectively because of a presumably aberrant value for total dry weight at harvest 5 (cf. Williams, 1939, p. 125). This being so, values of E_W and E_P for the composite harvest-interval 4–6 have been calculated by the usual formula, and are included in parentheses in Fig. 2. The very low value of E_W for harvest interval 6–7 is attributable to factors associated with senescence; these include the rapid disintegration of leaf proteins. As in the case of wheat, oats and Sudan grass the trend of E_P with time is related more closely with mean maximum temperature than with mean hours of bright sunshine per day for successive harvest intervals. The values of E_P for harvest intervals 4–5

TABLE 2.

Harvest.	L (cm.)	F_A (sq. mm.)	F_W (mg.)	F%	Mean area per cell. (sq. μ)	No. of cells per section.	Index of lignification. (p.c.)
1	21·3	0·042	15·1	43·9	—	—	nil
2	44·1	0·108	72·4	52·1	—	—	,,
3	75·4	0·209	236·8	65·8	—	—	,,
4	97·8	0·215	315·6	55·3	—	—	,,
5H	121·5	0·363	658·6	63·8	448	895	*
6H	124·4	0·408	758·4	60·0	513	842	9·16
7H	119·6	0·492	881·2	64·4	493	1,038	11·13
8H	125·0	0·466	872·9	66·2	471	1,030	11·76
5L	106·8	0·314	501·8	60·3	435	793	*
6L	110·1	0·337	557·1	55·2	444	807	7·18
7L	110·3	0·318	523·9	50·1	461	730	9·55
8L	108·6	0·339	550·7	53·1	413	865	8·08

H = high moisture. L = low moisture. * Lignification first observed.

[2] Expressed as percentage protein-N on dry weight basis in Fig. 2.

and 5–6 are greatly depressed by the low moisture treatment, their mean being only 40 p.c. of the value for the interval 4–6 with high moisture treatment. These effects are to be expected, since E_p is an approximate measure of the rate of carbon assimilation.

We are now in a position to interpret the observed effects of treatment on R in terms of the three factors discussed above. The depression in R with low moisture treatment is attributable to the depression in E_p, for the effects of treatment on leaf weight ratio and relative protein content tend to be in the opposite direction. The reversal after harvest 6 is clearly due to the marked increases in the latter factors with low moisture treatment.

ANATOMY OF THE FLAX STEM AND FIBRE BUNDLES.

Hector (1936) gives a comprehensive review of existing knowledge about the anatomy of the flax plant. Briefly, the chief structures of the flax stem as seen in cross section are a well defined epidermis, a narrow cortex and an endodermal layer surrounding the pericycle and bundles of pericyclic fibres; on the inner side of the latter are groups of phloem cells which are separated from the xylem by thin-walled cambial tissue (see Fig. 4, *2*, *4* and *6*). The parenchyma of the pith breaks down as the stem matures and only remnants of it remain attached to the protoxylem.

The distribution of some of these structural elements throughout the length of a mature stem is illustrated diagrammatically in Fig. 3. Jakovlev (1923, p. 288) presents data which, however, are not altogether in accord with those given here; this may be associated with differences of variety and of environment. Except for section 1 (Fig. 3), the cross-sectional area and hence the diameter of the stem is constant up to section 5; thereafter the area decreases continuously. As might be expected, the amount of xylem decreases continuously from the base of the stem upwards, but the pith, which is very small at section 1, shows a maximum near section 4. Numerical data showing the variation in total area of fibre cells and in other attributes of these cells are presented in Table 3. The number of fibre cells per section ranges from 250 (section 1) to approximately 1,150 (section 6), but is fairly constant for sections 3–9 inclusive. Associated with these variations in number there are striking differences in the mean areas of individual fibre cells, and in the lower sections (particularly section 1) the cell lumina are abnormally large. The number of fibre bundles per section is fairly constant, thus confirming the finding of Tammes (*loc. cit.*).

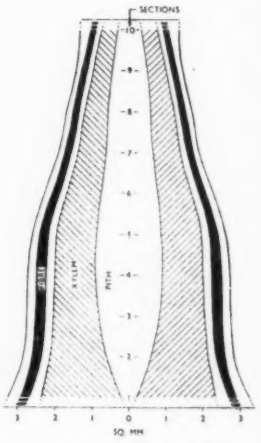

Fig. 3. Diagram showing the distribution of fibre in relation to other structural elements in a mature flax stem. The cross-sectional areas of the various elements have been plotted horizontally for the 10 sections of Table 3.

Considerable confusion exists in the literature concerning the origin of the fibre cells. Davis (1923) says that they are located in the cortex, whereas Hector (1936) calls them phloem fibres. Robinson (1933) presents fairly conclusive evidence for their pericyclic origin, and this view is accepted by Eames and MacDaniels (1925), Anderson (1927) and Alexandrov *et al.* (1932). The confusion may have arisen from the misuse of the term bast, which technically is synonymous with phloem; in commerce, fibres derived from the cortex, pericycle and secondary phloem have all been styled "bast" fibres. Anderson states that in flax the fibre cells are differentiated from the meristem of the stem tip and lie just inside the endodermis, but he is not very precise concerning the later development of the fibre bundle. From his photomicrographs (Plate XIX, Figs. 2–4) and from those of this paper (especially Fig 4, *2*, *4* and *6*), it seems clear that the outer layer of cells in each bundle is the first to develop the characteristic thickening of the cell walls, whereas the remaining cells appear large, thin-walled and hyaline. Normally the maturation of the cells takes place progressively towards the inner boundary of the fibre bundle until, at maturity, all the original cells are fully developed. The actual process of cell wall thickening in flax fibres has been studied in detail by Anderson (1927) and need not be considered here. The photomicrographs (Fig. 4, *2*, *4* and *6* and Fig. 5, *1* and *5*) constitute, with certain reservations, an ontogenetic series demonstrating progressive stages in the formation of the fibre bundle under conditions of optimal water supply. It must be remembered, however, that the variation in the size and other attributes of mature bundles is considerable, and that from the point of view of the potential number of fibre cells per bundle, only *4* and *6* of Fig. 4 and *1* and *5* of Fig. 5 are comparable (see methods). The latter series demonstrates very clearly that the number of fully developed cells per bundle increases with time.

The effect of the low moisture treatment on the anatomy of the fibre bundles at maturity is illustrated in *4* and *6* of Fig. 5, where the number of fully developed cells per bundle is seen to be greatly reduced by treatment. This reduction in number is doubtless due to an interruption

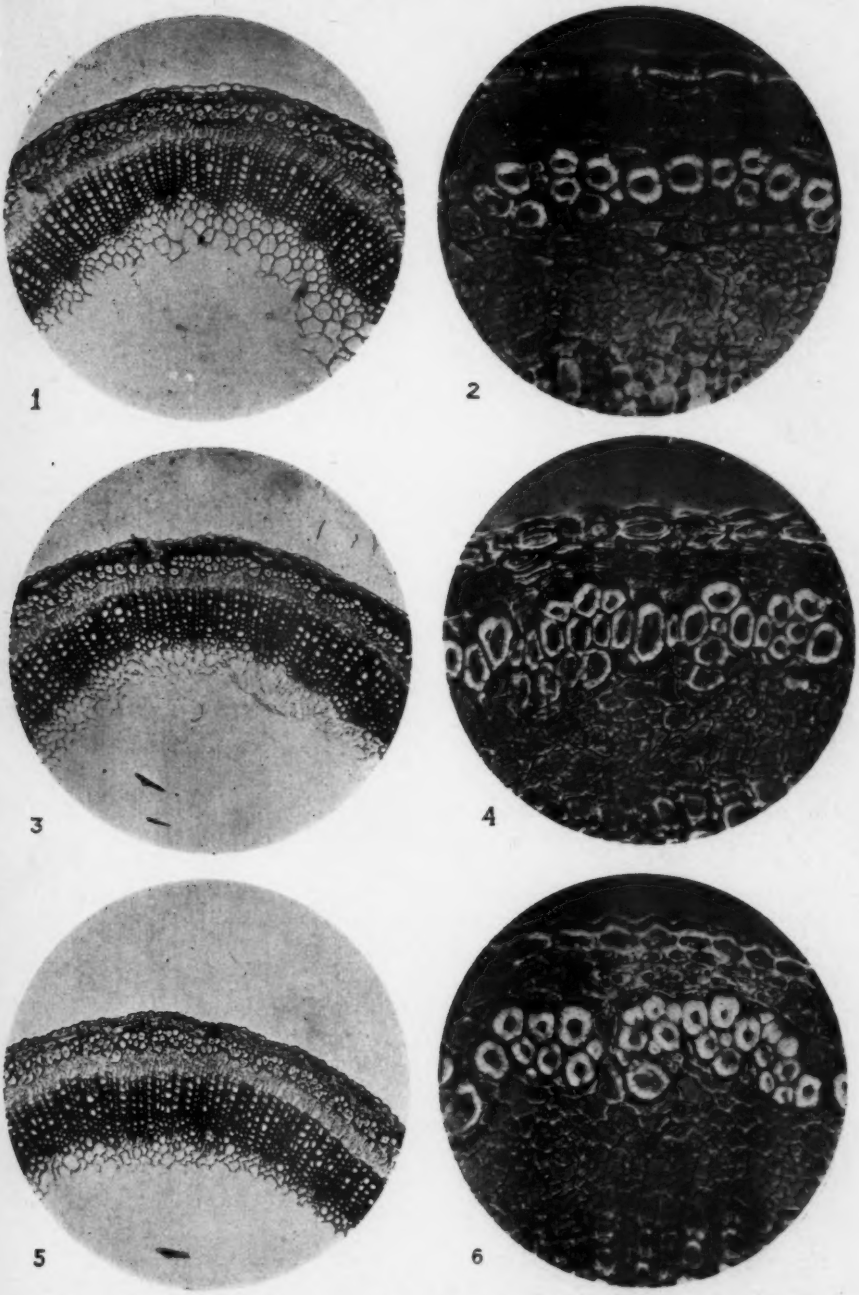

Fig. 4. Cross sections of the stem. *1.* Harvest 2. × 40. *2.* Harvest 2. × 200. *3.* Harvest 3. × 40. *4.* Harvest 3. × 200. *5.* Harvest 4. × 40. *6.* Harvest 4. × 200.

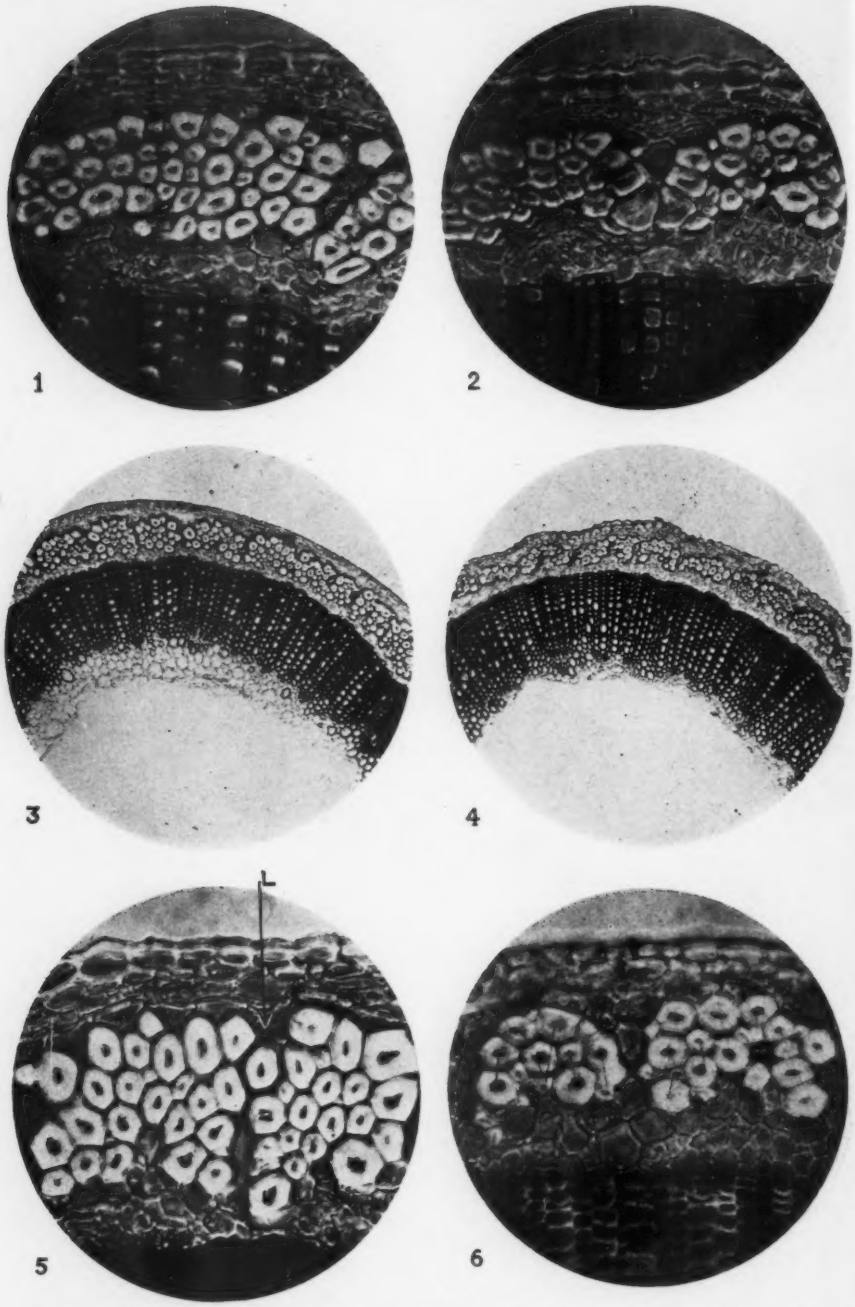

Fig. 5. Cross sections of the stem. *1.* Harvest 5, high moisture treatment. × 200. *2.* Harvest 5, low moisture treatment. ×200. *3.* Harvest 7, high moisture treatment. × 40. *4.* Harvest 7, low moisture treatment. × 40. *5.* Harvest 7, high moisture treatment, L = lignified fibre cell. × 200. *6.* Harvest 7, low moisture treatment. × 200.

in the normal development of the bundle, and examination of a large number of photomicrographs for harvests 7 and 8 tended to confirm this view. Although these stems were mature by the usual external criteria, many of the cells on the inner side of the bundles remained thin-walled (Fig. 5, *6*). These effects of low moisture treatment may be interpreted in terms of the relative deficiency of carbohydrates inferred from the depression in E_P.

TABLE 3.

Distribution of fibre in a mature flax stem.

Sect. No.	Height of sect. above cotyledons (cm.)	Total area of fibre cells (sq. mm.)	No. of fibre cells per section.	No. of bundles.	Mean area of indiv. fibre cells. (sq. μ)	Area "true" fibre p.c. total fibre (approx.)	Index of fibre percentage $\frac{\text{fibre}}{\text{fibre + xylem}} \times 100$ This exp.	Jakovlev.
10	92·5	0·274	612	24	448	95	23·9	29·0
9	82·5	0·325	876	28	371	"	24·6	32·5
8	72·5	0·358	1,058	32	338	"	22·3	30·8
7	62·5	0·351	1,054	34	333	"	19·1	29·5
6	52·5	0·413	1,157	36	357	"	18·7	30·5
5	42·5	0·500	1,071	35	467	"	19·1	29·0
4	32·5	0·541	1,129	32	479	"	20·1	27·0
3	22·5	0·566	927	33	611	"	18·7	21·5
2	12·5	0·526	585	32	899	90	14·9	19·5
1	2·5	0·442	250	20	1,768	80	9·5	12·0

LIGNIFICATION.

Lignification of flax fibre cells has been recorded by Tammes (1907), Anderson (1927), Schmalfuss (1936) and others, and in the present experiment it was first observed at harvest 5. Only isolated cells or groups of cells show lignification, and this is restricted to the middle lamella and secondary wall of these cells; Anderson claims that lignification rarely occurs in the tertiary wall. A single lignified fibre cell is indicated in *5* of Fig. 5. The index of lignification (percentage of lignified cells) shown in Table 2 is derived from data of sections taken 5 cm. below the centre of the stem. It is probable that lignification increased most rapidly between harvests 5 and 6; thereafter the data suggest only a slow increase with time. Since only a small part of the wall of each cell is affected, it is clear that the index grossly overestimates the percentage of lignin by weight. It is also probable that the rate of increase in lignin production with time is greater than that indicated by the data, as no adequate measure could be made of the increase in lignification within the individual cells affected. Anderson (1927) reports that lignification is most widespread in the fibres at the base of the stem, and this was confirmed by an examination of the ten stem-sections discussed above.

In the present experiment the index of lignification is consistently lowered by low moisture treatment (harvest 6–8), and may in part be attributed to the delayed development noted above. Schmalfuss (1936) claims to have shown an increase in lignification with low moisture treatment, but his data are open to criticism. His claim is apparently based on the mean values for fifteen manurial treatments run at his two moisture levels. The difference between these values was small, and within individual manurial treatments the variations with water supply were large and inconsistent. Moreover, the two moisture levels rarely produced an appreciable difference in yield of dry matter, hence it is doubtful whether his low moisture treatment really affected the normal development of the plant.

QUANTITATIVE ASPECTS OF FIBRE DEVELOPMENT.

The data for length of stem (L) and area of "true" fibre (F_A) used in the estimation of F_W and $F_{\%}$ according to equations (1) and (2) are presented along with these latter quantities in Table 2, and graphically in Fig. 6. The primary data for these quantities (harvests 5-8 only) were subjected to analyses of variance. The following discussion is concerned with the effects of time and treatment on F_W and $F_{\%}$, and with certain factors relevant to these two quantities.

Total weight of fibre per stem (F_W). As might be expected the increase in F_W with time is, to a considerable extent, determined by the growth of the stem

as a whole (see Fig. 1). With the high moisture treatment, F_W increases up to harvest 7, there being significant increases for harvest intervals 5-6 and 6-7. With the low moisture treatment, however, there are no significant differences in the values of F_W after harvest 5. The depression in F_W with low moisture treatment is significant for harvests 5–8 and at maturity (harvest 7) this depression is of the order of 40 p.c., which is almost twice as great as that in the dry weight of the stem.

Although the total height of the plant continued to increase until the inflorescence was fully developed, L remained constant after harvest 5. Analysis of variance revealed that the interaction between time and treatment was not significant, but that L was significantly depressed by low moisture treatment. It should be pointed out that the plants of some of the pots suffered slight wind-damage during the course of the experiment, and this may have contributed to the variation in L.

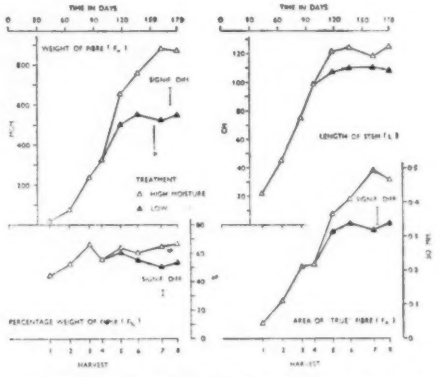

Fig. 6. Curves showing the effects of two moisture treatments on the quantities F_W, $F_{\%}$, L and F_A. P indicates the generally accepted time of pulling.

The area of "true" fibre, F_A, is determined by, (a) the number of fully developed fibre cells per section, (b) the cross-sectional areas of individual fibre cells, and (c) the areas of their lumina. In measuring F_A, only cells showing tertiary thickening were included, and, in view of what has been said concerning the nature of the development of the fibre bundle, it is clear that factors (a) and (c) are the more important determinants of the rapid increase in F_A with time. The effect of treatment on F_A is highly significant and it is also of interest to note that, with low moisture treatment, this quantity remains practically constant after harvest 5. Data for mean area per fibre cell and number of fibre cells per section at harvests 5–8 are presented in Table 2. Although these data are somewhat erratic, both show constant depressions with low moisture treatment. It is possible that these two factors are interrelated since, with high moisture treatment, the cells on the inner side of each bundle tend to be slightly larger than those of the outer layers: the former are those which do not develop with low moisture treatment, hence one would also expect a decrease in the mean area per fibre cell with this treatment.

Although both L and F_A contribute almost equally to the rapid increase in F_W up to harvest 5, the continued increase in the latter quantity with high moisture treatment beyond this harvest is solely attributable to a corresponding increase in F_A.

Percentage weight of fibre ($F_{\%}$). Apart from a small initial rise, this quantity does not vary greatly with time; in comparison with values obtained for commercially processed straw, however, they appear high.

Commercial yields of line fibre are extremely variable, but values of 25 p.c. for total fibre are considered good. Several reasons are suggested to explain the apparent discrepancy between calculated and commercially obtained results. These are: (a) $F_{\%}$ is overestimated by approximately 10 p.c. (see above); (b) $F_{\%}$ is expressed on an oven-dry basis, whereas commercial yields are based on air-dry straw, which contains at least 10 p.c. moisture; (c) field grown straw includes the deseeded inflorescence, whereas this fraction is not considered in the estimation of $F_{\%}$; (d) field grown flax is usually contaminated with weeds to a greater or lesser extent; (e) there are unavoidable losses during retting and mechanical processing.

When it is considered that the index of fibre percentage used in Table 3 (ratio of fibre area to the sum of the areas of fibre and xylem) gives an average of approximately 20 p.c. for the whole stem, and that the density of the fibre cells must be several times that of the xylem, a value for $F_{\%}$ as high as 50 p.c. is not excessive. Moreover, the data of Herzog quoted by Schmalfuss (1936) are of the same order as those presented here.

It has already been pointed out that the effect of treatment on F_W is relatively greater than that on the dry weight of the stem; from this it follows that $F_{\%}$ must be depressed with low moisture. Reference to Fig. 6 shows that this depression becomes significant at harvest 6 and is highly significant at maturity. The practical implications of this effect will be discussed below.

PRACTICAL SUGGESTIONS.

The results of this experiment are clearly of importance in relation to field practice, but the limitations imposed on direct inference by the difference between field and glasshouse conditions must be kept in mind. With adequate moisture supply the total yield of fibre increased up to harvest 7 (Oct. 14), but the generally accepted time for harvesting was reached during the week ending Oct. 7, that is, when the seeds began to rattle in the bolls, when the leaves had dried off for about three-quarters of the length of the stem and when the latter had changed from green to yellow for about the same distance. The loss in yield incurred by harvesting at this stage rather than at full maturity is slight, and is offset by the quality of the fibre, which is largely determined by the degree of lignification. Lignification increases with age and is undesirable in practice, since the fibres become difficult to separate from the wood during retting, and silkiness, lustre and elasticity are also lost.

An important result of the investigation is that plants grown with an adequate water supply give a much higher percentage weight of fibre than plants subjected to partial drought after flowering. This implies that straw produced under dry finishing conditions will not yield the same quantity of scutched fibre as straw produced under good finishing conditions, and that the weight of straw is not an accurate index of the weight of fibre it contains. In this connection, the results (Table 4) of two experimentally retted samples of straw are of interest. The relative difference in percentage line fibre for the two moisture treatments is of approximately the same order as that between the values for $F_{\%}$. Although both were of good strength, the high moisture sample proved to be stronger than the low moisture sample; the latter, however, was graded higher than the former. A possible explanation for this might be the delayed maturity with low moisture treatment. This suggests that the plants of the high moisture treatment should have been harvested earlier for this test.

TABLE 4.

Results for experimentally retted straw from harvest 7 (W. L. Greenhill).

Treatment.	Line fibre p.c.	Strength kg./sq. mm.
High moisture	22·8	82·1
Low moisture	18·8	72·8

The fact that the low moisture treatment has such a marked effect on fibre development must inevitably limit flax production to areas with a sufficiently long growing season. In some districts it would be necessary to sow sufficiently early to ensure that growth was completed before the onset of dry conditions. Field trials at the Waite Institute (Pike, 1941) have shown that early sowing is essential for maximum yields of straw. Further investigations of the effect of early and late sowing on the fibre production of two varieties of field grown flax are in progress.

SUMMARY.

The present study of the flax plant (*Linum usitatissimum*, L) concerns the physiology of growth and the developmental anatomy of the fibre bundles, and, after flowering, the effects of low moisture treatment are considered.

The growth of the whole plant was depressed by low moisture treatment, and the growth analysis revealed that this was due to a depression of the net assimilation rate (protein basis). The flax plant was shown to have a very high stem weight ratio.

Anatomical studies showed that the outer layer of cells in each bundle was the first to develop the characteristic thickening of the fibre cells. Normally, this process takes place progressively towards the inner boundary of the fibre bundle, but with low moisture treatment the inner cells fail to thicken.

Lignification of the fibre cells was first noted soon after flowering; thereafter it increased with time, but was less pronounced with low moisture treatment.

At maturity, low moisture treatment depressed the yield of fibre by 40 per cent., and the percentage weight of fibre was depressed by over 20 per cent.

The practical implications of the data are briefly considered.

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STUDIES ON SULPHHAEMOGLOBIN

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The problem of the chemical nature of sulphhaemoglobin is at present in a confused state. While some authors claim that sulphhaemoglobin is a derivative of protohaematin, others believe that it is a bile pigment haematin derivative. The difficulty of ascertaining its structure is largely due to the fact that sulphhaemoglobin cannot be obtained free from haemoglobin without the formation of other products. Michel (1938) has shown that sulphhaemoglobin is formed by the combined action of hydrogen sulphide and hydrogen peroxide on haemoglobin. By the combined action of hydrogen peroxide and reducing substances, e.g., ascorbic acid, as well as by coupled oxidation, the porphyrin ring of haemochromogens and of haemoglobin is split with the formation of bile pigment haematin compounds, Lemberg (1935), Lemberg *et al.* (1938a). Haemoglobin is thus transformed into choleglobin, Lemberg *et al.* (1938b, 1939a, 1941a, b, c.). It was therefore tempting to assume that sulphhaemoglobin belongs to the same class of compounds, and this view was held by Barkan (1938). Barkan found that a sulphhaemoglobin solution, obtained by the successive action of hydrogen sulphide and oxygen on haemoglobin or haemolyzed blood, contained "easily detachable iron" up to 30 p.c. of the total haemoglobin iron. Jung (1939) also concluded that sulphhaemoglobin is usually "verdohaemochromogen", this term being evidently used by Jung to cover bile pigment haematin compounds in general. The paper of Jung has been accessible to us only as an abstract. v. Restorff (1940), referring to this paper, used the term verdohaemochromogen for sulphhaemoglobin observed after sulphanilamide treatment in the blood of rats.

The results of other authors, however, are in disagreement with this hypothesis. Both Drabkin and Austin (1935) and Michel (1938) observed that by alkali and pyridine the absorption band of sulphhaemoglobin in the orange part of the spectrum is made to disappear and a strong band of protohaemochromogen appears which they found to possess between 70 and 96 p.c. of the original strength. Barkan attempts to explain this observation as due to protohaemochromogen formation from admixed unaltered protohaematin and nitrogenous compounds set free from a non-combining denatured protein. This explanation is hardly convincing and fails entirely to explain the quantitative measurements reported below. Haurowitz (1940) found only small amounts of easily detachable iron in his sulphhaemoglobin preparations. Splitting with acid yielded sulphur-containing porphyrins and not bile pigments.

Reduced choleglobin has an absorption curve similar to that of sulphhaemoglobin, but can be distinguished from the latter by its behaviour to alkali or to carbon monoxide (Lemberg *et al.*, 1939a). Lemberg (1939b) observed the formation of small amounts of bile pigment by the treatment of sulphhaemoglobin solutions with acids and also some increase of the easily detachable iron in sulphhaemoglobin formation. In these papers as well as at the Zürich International Physiology Congress (1938) Lemberg *et al.* have, nevertheless, clearly voiced their disbelief

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in the assumption of the bile pigment haematin nature of sulphhaemoglobin (compare also Lemberg, 1938). It appeared possible to us that the porphyrin may be opened without the removal of the bridge carbon atom, so that a reclosure of the porphyrin ring by alkali can occur. This hypothesis is not very satisfactory for stereochemical and chemical reasons. We shall show in this paper that the discrepancies between the observations of the various authors are to be explained by the formation of choleglobin or a choleglobin-like bile pigment haematin compound accompanying the formation of sulphhaemoglobin.

EXPERIMENTAL.

Spectroscopic Observations.

Sulphhaemoglobin differs from choleglobin in its behaviour to alkali in the presence of sodium hyposulphite. The band of choleglobin in the orange part of the spectrum is not dispersed by this treatment, but is shifted to 619 mμ (cholehaemochromogen). If solutions of sulphhaemoglobin are treated in the same way the band in the orange becomes much weaker or disappears entirely and strong bands of protohaemochromogen appear. It is essential that the solution should be more acid than pH 7 before addition of $Na_2S_2O_4$ and alkali. If this precaution is not taken, cholehaemochromogen may be obtained from haemoglobin owing to the formation of H_2O_2 from $Na_2S_2O_4$ in alkaline solutions. H_2O_2 acts rapidly on reduced haemoglobin but much more slowly on reduced haemochromogen. A safe way of carrying out the test for the presence of choleglobin is to add hyposulphite and alkali, while coal gas is bubbling through a solution already saturated with the gas. Cholehaemochromogen is thus transformed into CO-cholehaemochromogen with an absorption band at 628 mμ, while no band can be observed with fresh solutions of oxyhaemoglobin. Again the sulphhaemoglobin band of some samples of sulphhaemoglobin disappears entirely, while a weak band at 628 mμ is found in other samples. Table 1 reveals that no choleglobin can be discovered in samples of sulphhaemoglobin from human clinical cases and in haemoglobin solutions exposed to H_2S for a short period, while choleglobin in varying amounts is found in other preparations of sulphhaemoglobin and in a sample of rat's blood containing sulphhaemoglobin after dosing with sulphur and acetanilide. Occasionally a double band with maxima 628 mμ and 618 mμ is seen after action of CO on sulphhaemoglobin solution. The first maximum is due to CO-choleglobin, the second to CO-sulphhaemoglobin.

TABLE 1.

	Sulphhaemoglobin caused by	Absorption bands of Sulphhaemoglobin.	Absorption bands of Cholehaemochromogen.
Clinical case of microcytic anaemia	1 heaped teaspoonful of sulphur daily for 2 years, "gymnasium" therapy	+	Absent
Clinical case septicaemia	Sulphanilamide therapy	+++	Absent
Clinical case cyanosis	Phenacetin misuse	++	Absent
Rat's blood	Sulphur and acetanilide feeding	+++	+
	Sulphhaemoglobin produced by		
Human oxyhaemoglobin (lysed cells, destromatized with alumina cream) 10 c.c., 5 p.c.	0·2 c.c. H_2S water, 15 mins.	+	Absent
	0·5 c.c. H_2S water, 15 mins.	+++	+
	0·5 c.c. H_2S water, 12 hours	+++	++
Crystalline horse oxyhaemoglobin 10 c.c., 5 p.c.	H_2S gas, 90 seconds	++	+
	H_2S gas, 3 mins.	++++	++
	Ammonium sulphide, 0·2 c.c. 10 p.c., 15 mins.	++++	+++

The impression is gained from the observations summarized in Table 1, that choleglobin or a spectroscopically very similar substance is present in many preparations of sulphhaemoglobin in varying proportions, while it is missing in clinical samples.

Spectrophotometric Analysis.

If alkali transforms sulphhaemoglobin as well as haemoglobin into protohaemochromogen, a spectrophotometric analysis of mixtures of sulphhaemoglobin, haemoglobin and choleglobin becomes possible, since a three-substance problem is thus reduced to two two-substance problems. The analysis of mixtures of haemoglobin and choleglobin from the CO-haemochromogen absorption curves has been worked out previously (cf. Lemberg *et al.* 1941b, 1942) and can be applied to our problem with the single difference that instead of the concentration of haemoglobin that of

haemoglobin plus sulphhaemoglobin is obtained. In order to eliminate the danger of slight cloudiness of the solutions, the optical density at 740 mμ, in a region where none of the compounds absorb strongly, is subtracted from the optical densities at the wavelengths of maximal absorption of CO-protohaemochromogen (570 mμ) and CO-cholehaemochromogen (630 mμ). The following equations hold:

$$(h + s) = 0{\cdot}115\,([\epsilon 570 - \epsilon 740] - 0{\cdot}45\,[\epsilon 630 - \epsilon 740])$$
$$ch = 0{\cdot}115\,([\epsilon 630 - \epsilon 740] - 0{\cdot}03\,[\epsilon 570 - \epsilon 740]),$$

where (h + s) is the concentration of protohaemochromogen derived from haemoglobin and sulphhaemoglobin, and ch the concentration of cholehaemochromogen in gm. per 100 c.c. The denaturation of haemoglobin was effected within a few seconds by 1 p.c. sodium hydroxide with human and horse haemoglobin, and with 2 p.c. sodium hydroxide for rats' haemoglobin.

Table 2 shows that the choleglobin content of some samples of sulphhaemoglobin solutions, notably those from clinical cases of sulphhaemoglobinaemia is nil or negligible, while some sulphhaemoglobin solutions particularly those prepared with ammonium sulphide or after prolonged treatment with hydrogen sulphide, contain large amounts of choleglobin. This confirms the opinion already held by Drabkin and Austin that sulphhaemoglobin is converted into protohaemochromogen and excludes Barkan's interpretation of the formation of protohaemochromogen.

Our results throw doubt on the validity of the deductions of the sulphhaemoglobin absorption spectrum from the absorption curves of mixtures of reduced haemoglobin and sulphhaemoglobin carried out by Drabkin and Austin. Their solutions must have contained varying amounts of choleglobin in addition to haemoglobin and sulphhaemoglobin. Fortunately the absorption curves of reduced sulphhaemoglobin and of reduced choleglobin are not very different. This explains why Drabkin and Austin failed to discover the presence of a third substance in their supposed sulphhaemoglobin-haemoglobin mixtures, and on the other hand it still makes it possible to use their method for an approximate determination, not of sulphhaemoglobin, but of the sum of sulphhaemoglobin and choleglobin. The use of reduced haemoglobin entails one experimental difficulty, which we overcome by the use of the CO-compounds. It is difficult to avoid adding with the diluent fresh oxygen, or hydrogen peroxide if $Na_2S_2O_4$-containing solution is used, and the resulting formation of additional sulphhaemoglobin and even more choleglobin from reduced haemoglobin, while CO-haemoglobin is much less readily attacked. At a reaction more acid than pH 7 and with sulphhaemoglobin solutions which do not require dilution for spectrophotometric measurement, the method of Drabkin and Austin yields reproducible results and was used by us to determine the hitherto unknown extinction coefficient of CO-sulphhaemoglobin. The following constants for CO-sulphhaemoglobin containing 1 gm. per litre with a small content of choleglobin (20 p.c.) were thus established: $\epsilon 617\ m\mu - \epsilon 740\ m\mu = 0{\cdot}94$, $\epsilon 570\ m\mu - \epsilon 740\ m\mu = 0{\cdot}49$. The respective values for CO-choleglobin previously established are 0·85 and 0·45, sufficiently close to allow the use of one set of equations for the sum of CO-sulphhaemoglobin and CO-choleglobin. The following equations are derived from the extinction coefficients for CO-sulphhaemoglobin and CO-haemoglobin:

$$h = 0{\cdot}115\,([\epsilon 570 - \epsilon 740] - 0{\cdot}52\,[\epsilon 617 - \epsilon 740])$$
$$(s + ch) = 0{\cdot}11\,([\epsilon 617 - \epsilon 740] - 0{\cdot}04\,[\epsilon 570 - \epsilon 740])$$

where (s + ch) is the concentration of CO-sulphhaemoglobin plus CO-choleglobin and h that of CO-haemoglobin in gm. per 100 c.c.

The applicability of these equations is checked by comparing the total sum of pigments, (s + ch) + h, obtained from the absorption spectrum of the CO-haemoglobins with (s + h) + ch, obtained from the absorption spectrum of the CO-haemochromogens. The agreement was found to be very good in general, although occasionally the haemochromogen value was slightly lower (cf. columns 4 and 5 of Table 2).

Table 2 shows that it is impossible to prepare sulphhaemoglobin free from haemoglobin with hydrogen sulphide. The more sulphhaemoglobin a sample contains the greater its choleglobin content. On standing in the presence of oxygen the sulphhaemoglobin content remains unaltered, while the choleglobin content rises (cf. Nos. 11 and 12). Phenylhydrazine, while accelerating the formation of sulphhaemoglobin (cf. Nos. 17 and 21), leads finally to a product containing more choleglobin and the same amount of haemoglobin (cf. Nos. 14 and 15).

Ammonium sulphide yields products containing almost equal parts of sulphhaemoglobin and choleglobin. Even samples with a small sulphhaemoglobin content contain much choleglobin, while with H_2S initially only sulphhaemoglobin is produced. Samples obtained from clinical sulphhaemoglobinaemia showed a considerable amount of choleglobin in only one instance of extraordinary high sulphhaemoglobin content. In sulphhaemoglobinaemia produced in rats by feeding with a diet containing sulphur and acetanilide, however, one third of the abnormal pigment was choleglobin.

Table 3 gives the results of the experiments with rats in greater detail. The effect of the feeding of sulphur and acetanilide on the animals was uniform, except that the degree of anaemia varied considerably. During the washing of the cells with physiological saline its was observed that an abnormally extensive haemolysis occurred. If the cells containing sulphhaemoglobin and choleglobin were particularly fragile, the saline washings could contain a higher percentage of sulphhaemoglobin and choleglobin. The results in Table 3 show that this is not so. The plasma

TABLE 2.

No.	Sulphhaemoglobinaemia due to	Haemoglobin.	h + (s + ch).	(h + s) + ch.	In p.c. of total pigment: Sulphhaemoglobin plus choleglobin.	In p.c. of total pigment: Choleglobin.	Sulphhaemoglobin in p.c. of sulphhaemoglobin plus choleglobin.
1	Prolonged ingestion of sulphur[1]	Human cells	—	—	6·0	0·38	94
2	Prolonged ingestion of sulphur, one week later	,, ,,	—	—	5·4	0·30	94
3	Phenacetin[1]	,, ,,	—	—	9·0	0·3	97
4	Sulphanilamide therapy[1]	,, ,,	—	—	21	2·5	88
5	Ingestion of 2 p.c. sulphur and 1 p.c. acetanilide in food	Rat cells	—	—	31	10·2	68
	Sulphhaemoglobin produced by						
6	H_2S Water 45 secs.	Horse oxyhaemoglobin[2]	0·26	0·25	3·7	0·0	100
7	,, ,, 2 mins.	,,	0·295	0·295	3·0	0·0	100
8	,, ,, 10 mins.	,,	0·295	0·295	41	13·5	67
9	,, ,, 15 mins.	Human oxyhaemoglobin[3]	5·90	5·60	4·2	0·0	100
10	,, ,, 15 mins.	,,	5·88	5·91	9·0	1·35	85
11	,, ,, 15 mins.	,,	4·55	4·36	26	6·6	74
12	,, No. 11 after 12 hrs. standing	,,	4·28	4·18	28	9·0	68
13	,, Water 15 mins.	Human cells	4·61	4·22	14	2·1	85
14	,, ,, 30 mins.	,,	0·16	0·15	74	27	64
15	,, ,, 30 mins. + 0·02 p.c. phenylhydrazine hydrochloride, cf. No. 14	,,	0·19	0·16	73	30	59
16	H_2S Gas 1·5 mins.	Horse oxyhaemoglobin[2]	0·26	0·25	12	2·9	76
17	,, ,, 3 mins.	,,	0·25	0·25	10	0·0	100
18	,, ,, 2 mins.	,,	0·26	0·25	34	6·8	80
19	,, ,, 3 mins.	,,	0·75	0·7	38	15·4	59
20	,, ,, 3 mins.	,,	0·27	0·26	52	16·9	67
21	,, ,, 3 mins. with 0·05 p.c. phenylhydrazine hydrochloride, cf. No. 17	,,	0·24	0·24	53	26·1	57
22	Ammonium sulphide[4] in phosphate buffer pH 7·4 8 mins.	,,	0·30	0·30	25	10·5	58
23	,, 15 mins.	,,	0·29	0·30	36	18·0	50
24	,, 30 mins.	,,	0·29	0·29	33	17·1	48
25	Ammonium sulphide[4] unbuffered 35 secs.	,,	0·30	0·29	3·0	3·0	0
26	,, 2 mins.	,,	0·29	0·29	16	8·0	50
27	,, 4 mins.	,,	0·31	0·30	27	11·2	58
28	,, 30 mins.	,,	0·28	0·27	35	21·2	39
29	,, [5] 15 mins.	Human oxyhaemoglobin[3]	5·84	5·47	28	8·5	69

[1] Cf. Table 1.

[2] Solution of crystalline horse oxyhaemoglobin in water with a few drops 10 p.c. secondary sodium phosphate.

[3] Cells haemolysed and destromatized with alumina cream.

[4] Ammonium sulphide, 1 day old, added to concentration of 0·5 p.c.

[5] Ammonium sulphide, freshly prepared, added to concentration of 0·4 p.c.

also did not contain a higher content of abnormal pigments than the cells, while the choleglobin was slightly, but not significantly higher. Michel has tried in vain to separate sulphhaemoglobin from haemoglobin by crystallization, and our attempts to separate choleglobin from haemoglobin have likewise failed. It is therefore of interest that during the spontaneous crystallization of oxyhaemoglobin from the haemolysed rats' cells a good separation was observed. The oxyhaemoglobin crystals contained only one-third as much sulphhaemoglobin as the cell haemolysate and were practically free from choleglobin, while these pigments were enriched in the mother liquor almost twice.

TABLE 3.

	Total pigment in cells. gm. per 100 c.c.	Total pigment in blood gm. per 100 c.c.	Sulphhaemoglobin plus choleglobin in p.c. of total pigment.	Sulphhaemoglobin in p.c. of sulphhaemoglobin plus choleglobin.	Haemolysis in plasma.
Rat 1	25·9	—	31	68	—
Rat 4	28·3	15·8	24	73	(+)
Rat 5	25·9	6·5	20	62	++
Rat 6	25·9	12·9	25	69	+
Rat 7	25·7	11·9	23	47	++
Plasma (rats 4–7)	—	0·22	24	54	—
First saline washings of cells	—	0·28	24	62	—
Second saline washing of cells	—	0·26	21	64	—
Oxyhaemoglobin crystals	—	—	8·2	94	—
Mother liquor of crystals	—	—	45	66	—

Studies of the Ultraviolet Absorption.

The experiments summarized in Table 4 were carried out in a Hilger echelon cell. A stroma-free oxyhaemoglobin solution was prepared by laking rabbit cells, keeping the solution in a refrigerator at pH 5·8 and centrifuging. The pH was then brought back to 6·5 for the determination of the absorption of CO-haemoglobin. H_2S shifted the pH slightly to the acid side (6·1). Transformation into CO-haemochromogens was effected by adding to 0·6 c.c. of the CO-haemoglobin solution an equal volume of 10 p.c. alcoholic ammonia followed by 0·2 c.c. sodium hyposulphite solution in strong ammonia (2 mg. $Na_2S_2O_4$) and 2·2 c.c. of CO-saturated water.

TABLE 4.

	$10^{-3}\,\epsilon$ 415 mμ mol. of CO-haemoglobins.	$10^{-3}\,\epsilon$ 415 mμ mol. of CO-haemochromogens.	P.c. sulphhaemoglobin plus choleglobin.	P.c. choleglobin.	Sulphhaemoglobin in p.c. of sulphhaemoglobin plus choleglobin.
Before action of H_2S	157	125	—	—	—
H_2S gas, 2 mins.	100	122	36	2·5	93
H_2S gas, 10 mins.	94	125	40	0	100
The same after standing over night open to air	78	99	50	21	58

CO-choleglobin does not possess a Soret band and its absorption in this region is very low (Holden and Lemberg, 1939). The percentages of conversion of haemoglobin into sulphhaemoglobin have been calculated assuming that CO-sulphhaemoglobin does not possess a Soret band either. They agree well with those found by the spectrophotometric analysis in the visible. The increase of the Soret band on transformation of the CO-haemoglobin into CO-haemochromogens confirms the transformation of sulphhaemoglobin into protohaemochromogen. After long standing a greater choleglobin content is again found by the less complete recovery of the Soret band of the haemochromogen. The results differ from those obtained by spectrophotometric analysis in the visible only by the smaller figures for the choleglobin content of fresh sulphhaemoglobin. As is shown in Table 2 this varies a good deal with the method of preparation, and small experimental differences, e.g. the oxygen content of the H_2S gas or pH, or species specificity may be the cause. It has also to be kept in mind that the analysis of the ultraviolet absorption gives only approximate values, since it neglects the absorptions of CO-sulphhaemoglobin and CO-choleglobin in the ultraviolet, which are certainly weak, but probably not negligible.

Does Sulphhaemoglobin Yield Bile Pigment on Splitting with Acids?

If a solution of sulphhaemoglobin is treated with glacial acetic acid and the mixture extracted with ether in the manner described by Lemberg *et al.* (1941b) biliverdin or biliviolin could always be isolated by extraction of the ether with hydrochloric acid. It was, however, observed that fresh samples of sulphhaemoglobin, prepared by relatively short action of H_2S water on oxyhaemoglobin, yielded much smaller amounts of bile pigment than samples obtained by prolonged action of hydrogen sulphide and air. Since erythrocytes and oxyhaemoglobin solutions yield traces of bile pigment, derived from admixed bile pigment haemoglobin, and since choleglobin yields bile pigment, only quantitative determinations can answer the question whether sulphhaemoglobin itself yields bile pigment. The choleglobin content also explains the increase of easily detachable iron.

As a sample of sulphhaemoglobin with small choleglobin content we chose the cells of the clinical case No. 1 in Tables 1 and 2. Isolation and determination of bile pigments were carried out with the method described by Lemberg and Legge (1942). 1 c.c. of erythrocytes yielded 14 μg. of bile pigments. If we subtract the 5 μg. usually found in cells without noticeable choleglobin content, 9 μg. were derived from sulphhaemoglobin or choleglobin. 1 c.c. of these cells contained 18·4 mg. sulphhaemoglobin and 1·2 mg. choleglobin. According to our previous experiences (11 p.c. of choleglobin split to bile pigment and 52 p.c. recovered) 1·2 mg. choleglobin yield at least 2·4 μg. if bile pigments; no more than 6·6 μg. can thus be derived from sulphhaemoglobin. Assuming an average recovery of 52 p.c. of the bile pigment by our method of isolation, no more than 2 p.c. of the sulphhaemoglobin can be split to bile pigment. This makes it very unlikely that any bile pigment is derived from sulphhaemoglobin. The slight excess of bile pigment over that expected from choleglobin is probably due to the presence of biliverdin in the cells, which had previously been observed in rabbit cells containing choleglobin after treatment with phenylhydrazine (Lemberg and Legge, 1942).

Similar results were obtained with the cells of rats after feeding with sulphur and acetanilide. These are summarized in Table 5. In view of the large choleglobin content of these cells, the presence of more free biliverdin could be expected.

TABLE 5.

	Bile pigment found per 1 c.c. of cells.	Excess bile pigment per 1 c.c. of cells.	Maximum splitting of sulphhaemoglobin to bile pigment.
			P.c.
Rat 1	103	47	4·8
Rat 4	100	58	6·4
Rat 6	94	49	6·0
Rat 7	73	5	0·9

DISCUSSION.

Our results indicate that "sulphhaemoglobin" is generally a mixture of sulphhaemoglobin and choleglobin. The former is a protohaematin derivative, reconvertible into protohaemochromogen and not yielding bile pigments on splitting with acids. The latter is a bile pigment haematin compound yielding biliverdin and biliviolin on splitting with acids, and not reconvertible into protohaemochromogen. The more complete is the transformation of haemoglobin into sulphhaemoglobin, and the longer "sulphhaemoglobin" is allowed to stand in the presence of atmospheric oxygen, the more choleglobin becomes admixed with sulphhaemoglobin. This explains the divergencies of the various authors. Drabkin and Austin as well as Michel studied products of incomplete conversion of haemoglobin into sulphaemoglobin by hydrogen sulphide, avoiding an excess of oxygen. Barkan treated his haemoglobin solutions alternatively with H_2S and oxygen for a long period.

The fact that the clinical samples of sulphhaemoglobin contained less choleglobin may be due to the milder conditions of formation of sulphhaemoglobin *in vivo*. It is possible that even *in vivo* sulphhaemoglobin formation is always accompanied by choleglobin formation, but that choleglobin is removed by its decomposition into biliverdin, while sulphaemogoblin persists for weeks. This

would appear to be supported by the fact that in the rats, with a strong formation of sulphhaemoglobin still in progress, choleglobin and biliverdin were found in the cells. Acetanilide fed to the rats may, however, have a dual action, catalytic acceleration of sulphhaemoglobin formation and direct formation of choleglobin.

SUMMARY.

Sulphhaemoglobin is a protohaematin compound reconvertible into protohaemochromogen. By acids it is not split into bile pigments.

Artificially produced samples of sulphhaemoglobin generally contain 20-50 p.c. of choleglobin, a bile pigment haematin compound. This is the source of the bile pigment and of the easily detachable iron obtained from sulphhaemoglobin preparations.

In clinical sulphhaemoglobinaemia in human beings little or no choleglobin was found. In rats fed with sulphur and acetanilide, however, choleglobin formed about one third of the abnormal pigments. By spontaneous crystallization of oxyhaemoglobin from haemolysed rat cells, sulphhaemoglobin and choleglobin could be enriched in the mother liquor.

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A CONSTANT-PRESSURE RESPIROMETER; DESIGNED FOR USE WITH MEDIUM VOLUMES OF YEAST SUSPENSION

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The well-known types of apparatus used for measuring changes in gas volume caused by respiring or fermenting cells are suitable for relatively small amounts of cell suspensions. These apparatuses fulfil their purpose in all those experiments which are concerned only with the investigation of the gaseous exchange. In experiments with fermenting or with respiring and fermenting yeast cells the necessity arose to investigate in addition the complete balance of the added substrate; this required larger amounts of material. If the current forms of apparatus are used for the study of the gaseous exchange in such cases, then the similar treatment of a larger amount of the same suspension in some other vessel is necessary. It is obvious that the reproduction of conditions in the second vessel is practically impossible.

The apparatus described in this paper allows the accurate measurement of fermentation and respiration with volumes of yeast suspension large enough for the necessary chemical analyses. It represents an improved and more convenient form of the apparatus described previously (Gottschalk, 1941) and has been used for one year with consistent and satisfactory results. Another advantage of the apparatus is that it can be built in any laboratory with standard equipment. The apparatus is of the constant-pressure type.

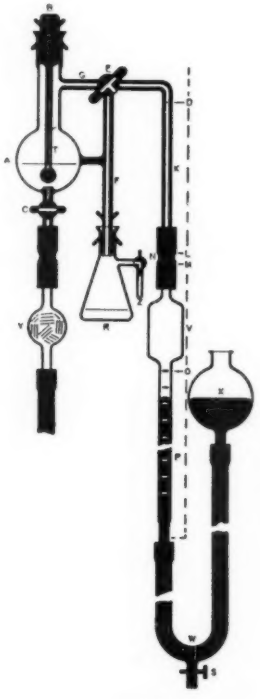

Fig. 1.

DESCRIPTION.

The apparatus consists of three parts (see Fig. 1): (1) the bulb A containing the yeast suspension and the substrate, (2) the absorption bulb R with the potash solution, (3) the gas-burette P with the mercury levelling bulb X.

The pyrex bulb A in which is placed the yeast suspension possesses two capillary outlet tubes. The lower tube (internal diameter = 0·5 mm.) allows equilibration with nitrogen or other gases and can be closed by tap C. The small filter Y retains any foreign matter inadvertently present in the gas which may block the capillary tube. Tube G (internal diameter = 2 mm.) connects to the other parts of the apparatus. The bulb A is closed by the ground glass joint B (standard taper B 14) to which is fused a thermometer T for checking the temperature of the yeast suspension. A small piece of platinum wire is sealed into the wall of T, as shown, to allow the suspension of a small glass container for the substrate. The volume of the bulb A including the outlet to L measured accurately by means of mercury, with B–T in position, is about 65 c.c.

The capillary three-way tap E connects the three parts of the apparatus. The capillary tube F (internal diameter = 2 mm.) leads into the absorption bulb R (volume = 50 c.c.) by means of a ground glass joint (size B 12); bulb R is filled with about 10 c.c. of 10 p.c. potassium hydroxide solution. Tap Z connects the bulb with the atmosphere.

A short piece of rubber pressure-tubing N of about 5 mm. bore and 13 mm. external diameter connects the capillary tube K (internal diameter = 2 mm.) with the gas-burette P. This

rubber tubing is carefully impregnated with hot vaseline (120° C) under pressure and coated with gold-size after assembly. The volume from D to L as well as the internal volume of the rubber tube N between L and M is determined. The latter volume should not exceed 0·1 c.c. The gas-burette P possesses a wider calibrated part V (volume about 15 c.c.) and the graduated part whose volume will depend on the maximum change of gas volume to be expected. In general a 25 c.c. graduated portion will suffice; with the help of a magnifying lens it is easy to read this burette to 0·03 c.c. The burette is calibrated with mercury. The levelling bulb X connected to the gas-burette by the pressure-tubing W contains mercury and enables adjustment to atmospheric pressure as well as efficient mixing of the whole gas space during an experiment. Tap S facilitates the removal of the mercury for cleaning; it is inadvisable to leave the mercury in the apparatus between the experiments. Mercury in contact with rubber tends to form a film which renders exact readings difficult.

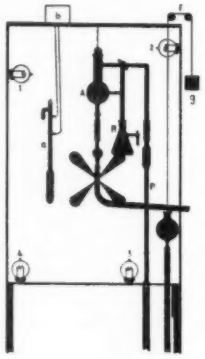

Fig. 2.

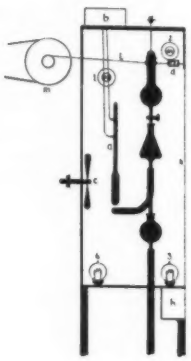

Fig. 3.

The apparatus is arranged in a thermostat-cabinet as shown in Figs. 2 and 3 (front and side elevations). The glass door (k) of the cabinet allows control of the apparatus during the experiment. The temperature is kept constant by means of a mercury contact type of regulator *a* in conjunction with a thermionic relay *b*. The four electric bulbs are used as heating elements and arranged to give uniform temperature when operated together with the fan *c*. The two bulbs (2, 3) nearest the apparatus are used initially to bring up the temperature and then switched off. The shaking system consists of a spring *d*, the wire *l* and the eccentric pulley *m* which is driven at the optimal rate by a rheostat-controlled electric motor. The levelling bulb is moved by means of pulley *f* and counterweight *g*. The electrical switches are mounted together on the board *h*. The ground glass joints and taps are firmly fixed and held in position by springs.

PROCEDURE.

Experiments under Anaerobic Conditions.

The yeast suspension is pipetted into bulb A. The small glass vessel containing the substrate is lowered into the bulb on a cotton loop and then, by introducing the thermometer T, suspended on the platinum hook. Tap E is turned to connect the three parts of the apparatus. The mercury is brought to level D. The inert gas (nitrogen, etc.) is bubbled through the yeast suspension by opening tap C and passed out through tube F which dips into water. Under anaerobic conditions R is replaced by a suitable container for water. While the gas is bubbling through the system the mercury is several times lowered and raised to replace by nitrogen the air present in the capillary tube above D. After equilibration tap C is closed and the system allowed to come to thermal equilibrium; this is facilitated by shaking gently. Then the mercury level is accurately adjusted to mark D, tap E turned through 180° and the substrate container dropped into the yeast suspension. The shaker is brought to optimal speed and the bulb X moved up and down to ensure a uniform gas mixture throughout the bulb A and the gas-burette. If one wishes to make readings of the volume of CO_2 produced at short intervals during an experiment the inert gas is drawn back into the gas-burette to mark O just before closing tap C. At the close of the experiment, i.e. immediately after taking the final reading, the thermostat-cabinet is opened, B removed and the yeast suspension quickly siphoned into a cooled centrifuge cup (Fig. 4).

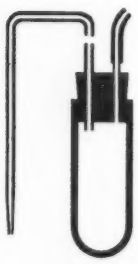

Fig. 4.

Experiments under Aerobic Conditions.

If air is used in these experiments tap C is kept closed; tap E is opened to connect bulb A with the gas-burette. Vessel R with tap Z open is thus cut off from the rest of the apparatus. The bulb X is lowered to near the end of the graduated part of the burette to take in air and finally the bulb A is prepared as described above. Thermal equilibrium is established and the initial reading taken. In order to ensure optimal oxygen supply throughout the experiment the partial pressure of oxygen must not be allowed to fall below 90 mm. Hg at any stage. This is accomplished by (a) adjusting the amount of yeast to the volume of air available, (b) shaking at about 180 oscillations per minute and (c) lifting and lowering the levelling bulb X several times per minute

to ensure a thorough mixing of the gases. After taking the final reading tap Z is closed and tap E turned clockwise 90°. The bulb A is emptied as described above. The carbon dioxide present in the space comprising the gas-burette and the tubing as far as tap E (calibrated volume) is then absorbed in the potash solution by raising and lowering X and simultaneously shaking the apparatus. The absorption is completed within about 20 minutes as judged by consistent readings.

If oxygen is used instead of air the system is at first equilibrated with the gas which enters through tap C and leaves through tap Z; the latter dips into water. Before finishing the equilibration oxygen is drawn into the graduated part of the gas-burette. Tap C is then closed and tap E turned through 180°. After thermal equilibrium has been established the initial reading is taken. The procedure as described for experiments with air is followed.

Each reading has to be corrected for the prevailing barometric pressure. The apparatus primarily being devised for the study of total balances of different substrates, in general only initial and final readings are required. Therefore we have not complicated the design by using a baro-compensator.

With a gas-burette whose graduated portion is less than 10 mm. diameter a correction has to be applied for capillary depression of the mercury (Cawood and Patterson, 1933). This small correction may be avoided by using an extra stationary manometer tube of the same diameter as the gas-burette (represented in Fig. 1 by the dotted line).

The graduations of the gas-burette can be read under the described conditions to 0·03 c.c. without difficulty; this introduces an error of ± 2 p.c. into the determination of the oxygen consumption and of less than ± 1 p.c. into the determination of the anaerobic carbon dioxide production. With the extra manometer tube fitted close to the gas-burette the readings can be made still more accurately.

CALCULATIONS.

V = volume of the bulb A, fitted with B–T to mark L;
Vc = volume of the capillary tube from E to L;
Vd = volume of the capillary tube from D to L;
Vs = volume of the substrate contained including volume of the substrate;
Vf = volume of the yeast suspension;
Vr = volume of tubing N between L and M;
Vb = volume of the gas within the gas-burette from M to the level of mercury at the beginning of the experiment;
T = absolute temperature of the thermostat;
α_{CO_2} = absorption coefficient of CO_2 in water at temp. T;
p, p′ = barometric pressures in mm. Hg at the times of the volumetric readings;
e = maximal tension of water vapor at temp. T.

1. *Anaerobic Fermentation.*

The initial volume Vg (N.T.P.) of the gas space above the yeast suspension in bulb A to mark D

$$Vg = \left[V - (Vd + Vs + Vf) \right] \left[\frac{p - e}{760} \cdot \frac{273}{T} \right]$$

Let the final volume of the gas space be V′g (N.T.P.). Included in this is the correction necessary if a solid substrate is used; its solution in the suspension causes the gas volume to contract by a corresponding amount.

The volume of CO_2 present in the gas space at the end of the experiment (N.T.P.)

$$V^{g}_{CO_2} = V'g - Vg;$$ the volume of CO_2 dissolved in the fluid (N.T.P.)

$$V^{f}_{CO_2} = \alpha_{CO_2} \cdot Vf. \frac{p' - e}{760} \cdot \frac{V'g - Vg}{V'g}.$$

Thus the total volume of fermentation CO_2 produced under anaerobic conditions (N.T.P.)

$$V^{N_2}_{CO_2} = V^{g}_{CO_2} + V^{f}_{CO_2}.$$

II. *Aerobic Conditions.*

Under aerobic conditions the yeast cells (baker's yeast) are simultaneously respiring and fermenting. The gasometric results give values for the total CO_2 produced and the O_2 consumed. The fermentation CO_2 is calculated as the difference between the total CO_2 produced and the oxygen consumed assuming that the R.Q. is unity, which is proved for glucose and other fermentable sugars as substrate.

The initial volume Vh (N.T.P.) of the gas space above the yeast suspension in bulb A, tubing N and the gas-burette:

$$Vh = \left[(V + Vr + Vb) - (Vs + Vf) \right] \left[\frac{p - e}{760} \cdot \frac{273}{T} \right].$$

Let the final volume of the gas space be V'h (N.T.P.). The volume of gas mixture analysed is Va (N.T.P.) and the residual volume after CO_2 absorption V'a (N.T.P.). Therefore the volume of CO_2 (N.T.P.) in the final volume V'h of gas mixture

$$V^{g}_{CO_2} = V'h \cdot \frac{Va - V'a}{Va}.$$

The volume of CO_2 dissolved in the fluid (N.T.P.)

$$V^{f}_{CO_2} = \alpha_{CO_2} \cdot Vf. \frac{p' - e}{760} \cdot \frac{Va - V'a}{Va}.$$

Total volume of CO_2 produced under aerobic conditions (N.T.P.)

$$V_{CO_2} = V^{g}_{CO_2} + V^{f}_{CO_2}.$$

The volume of oxygen consumed (N.T.P.)

$$V_{O_2} = Vh - V'h \cdot \frac{V'a}{Va}.$$

The volume of fermentation CO_2 produced under aerobic conditions (N.T.P.)

$$V^{O_2}_{FCO_2} = V_{CO_2} - V_{O_2}.$$

If instead of glucose, substances such as acetaldehyde, ethanol, acetic acid are used as substrates, the total volume of CO_2 produced under aerobic conditions represents respiration CO_2; in such cases the R.Q. can be directly determined.

SUMMARY.

An apparatus is described which allows the accurate measurement of fermentation and respiration with volumes of yeast suspension large enough to perform chemical analyses necessary for a complete balance of substrates added. The apparatus is of the constant-pressure type and has given consistent results within an accuracy of ± 2 p.c.

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THE CHEMICAL EQUATIONS OF THE PHYTOCHEMICAL REDUCTIONS BY FERMENTING YEAST CELLS AND THEIR RELATIONSHIP TO THE PASTEUR EFFECT

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In the last century it was already known that yeast possesses a strong reducing power. Systematic investigations on this subject were started when Lintner (1911) found that fermenting yeast reduced furfuraldehyde to the corresponding alcohol. Two years later Neuberg (1913) demonstrated the reduction of valeraldehyde to amyl alcohol and Lintner (1913) the reduction of choralhydrate to trichloro-ethanol. Since this time Neuberg and his collaborators (cf. review by Neuberg and Hirsch, 1923) have studied in detail this type of reaction which was termed "phytochemical reduction."

It has been established that many saturated aliphatic and aromatic aldehydes are reduced to the corresponding primary alcohols (formaldehyde, propionaldehyde, isobutyraldehyde, valeraldehydes, n-caproic aldehyde, n-heptaldehyde, glycol aldehyde, aldol, thioacetaldehyde, benz aldehyde, o-nitrobenzaldehyde, phenylacetaldehyde). The unsaturated aldehydes citronellal, citral and cinnamaldehyde are reduced to the corresponding olefinic alcohols. In recent years it has been shown by adding crotyl alcohol, cinnamic alcohol and sorbic alcohol to fermenting yeast that the ethylenic linkage (— CH : CH —) is also reduced (Fischer and Wiedemann, 1934). Another series of compounds which are reduced by fermenting yeast are substances with one or two keto groups. Ketones and diketones (e.g. acetyl methyl carbinol, methyl propyl ketone, hexyl methyl and methyl nonyl ketone, acetol as well as diacetyl and benzil) are reduced to the corresponding secondary alcohols. Finally it has been shown that fermenting yeast reduces nitrocompounds, both aliphatic and aromatic, to the corresponding amines. By far the highest rate of phytochemical reduction is attained by adding the reducible substance to a vigorously fermenting mixture of living yeast and sugar. In several cases, e.g. with d, l-valeraldehyde, aldol, diacetyl as substrates, laevorotatory amyl alcohol and optically active butylene glycols respectively are formed, indicating the enzymatic nature of the phytochemical reduction.

As to the mechanism of these reductions Neuberg (1923) suggested that they are effected by the "mobile hydrogen", which normally reduces the fermentation acetaldehyde to ethyl alcohol. In none of the numerous experiments, however, could an equivalent amount of acetaldehyde, thus protected from reduction to ethanol, be found among the end-products of fermentation (cf. Neuberg, 1923); sometimes no acetaldehyde at all was detectable (Fischer, 1934). The yield of the reduced product amounted in several instances to 80 p.c. of the theory. This experimental result together with the fact that the acid corresponding to the added aldehyde is not formed is definite proof that the production of the alcohol is not due to a dismutation of the aldehyde, a mechanism, moreover which would not be applicable to the reduction of simple ketones or of nitro compounds.

The above mentioned experiments were carried out with German brewery yeast and with compressed yeast. No steps were taken to ensure either complete absence of oxygen on the one hand or its optimal supply on the other.

In this paper the problem of the phytochemical reductions is subjected to a renewed investigation mainly from two aspects. Firstly, the phytochemical reductions were performed either under strictly anaerobic conditions or with an optimal supply of oxygen to the respiring and fermenting compressed yeast. Secondly, quantitative determinations of the reduced substances and of the products of alcoholic fermentation (in nitrogen) or of respiration plus fermentation (in air) were made. These balances demonstrate that the mechanism of the phytochemical reductions differs under anaerobic and under aerobic conditions and enable us to formulate the chemical equations of these reactions.

METHODS.

The experiments were carried out with the apparatus described by Rawlinson and myself in this Journal (1942). This apparatus permits the exact measurement of CO_2 production and O_2 consumption with a volume of yeast suspension large enough for the investigation of the chemical changes of the added substrate.

Fresh compressed baker's yeast was used for the experiments. The yeast was suspended in M/20 KH_2PO_4 solution; pH of the suspension at the beginning of the experiment was 4·3.

During the period of equilibration with the gas (either O_2-free nitrogen or air) and of thermal equilibration the glucose and the reducible substrate, the latter in a standard solution, were suspended in a small glass container in the bulb above the yeast suspension.

At the end of the experiment the yeast suspension was quickly siphoned into a cooled centrifuge tube, frozen and centrifuged as described. The dry weight of the yeast was determined as already described, but the temperature of the electric oven was 92° C. (Gottschalk, 1941).

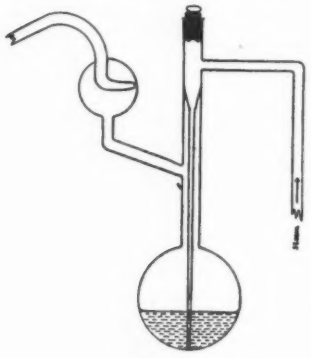


Fig. 1.

The acetaldehyde in the standard solution and the ethyl alcohol and acetaldehyde in the distillate from the suspension fluid of the yeast were determined as before (Gottschalk, 1941). In cases where the presence of acetyl methyl carbinol (acetoin) in the distillate interfered with the determination of acetaldehyde or ethyl alcohol a small correction was applied. From the investigations of Langlykke and Peterson (1937) the rate of distillation of acetoin from aqueous solutions is known. According to Stahly and Werkman (1936) one mol of acetyl methyl carbinol when oxidized by $K_2Cr_2O_7 + H_3PO_4$ yields 2 mols of acetic acid.

For the *determination of acetic acid* the following procedure gave satisfactory results.

10–12 c.c. of the clear supernatant fluid (after centrifuging off the yeast cells) were acidified with 1/10 of its volume of N. H_2SO_4 and put into a small distilling flask the arrangement of which may be seen from Fig. 1. The liquid in the flask was at first concentrated to half its original volume by heating the bulb in a glycerol bath the temperature of which was slowly raised to 145–148° C., the distillate being collected in a measuring cylinder surrounded by ice water. Then steam was passed in and a further 20 c.c. of distillate driven over. During the steam distillation the temperature of the glycerol bath was allowed to fall to about 130° C. By the end of the steam distillation the fluid in the distilling flask had thus been concentrated to ⅓ of its original volume. Titration was made with N/20 NaOH (freshly prepared each time from solid sodium hydroxide and standardized against N/20 HCl) with phenolphthalein as indicator. Tests with 2·0 c.c. N/20 acetic acid showed that by means of this method 95 p.c of the acetic acid added could be recovered in the distillate.

In the experiments with *diacetyl* as reducible substrate this diketone, left at the end of the experiment, was *determined* by the colorimetric method of Pien *et al.* (1937). The principle of this method is the comparison of the yellow colour produced in the distillate from the diacetyl-containing fluid by o-diamino benzidine hydrochloride with the colour given by a freshly prepared diacetyl standard solution. This method has been tested and found to be accurate for the estimation of small quantities of diacetyl by Cox and Wiley (1939). Their procedure was modified as follows: 7–8 c.c. of the clear supernatant fluid and 0·7–0·8 c.c. N. acetic acid were pipetted into a 50 c.c. distilling flask connected to a fractionating column. The outlet of the column forms the internal glass tube of a short Liebig condenser and then runs through a jacket filled with ice water; the end dips into a graduated test tube filled with 2–3 c.c. distilled water and packed in snow. The distilling flask, the fractionating column and the Liebig condenser are of one piece of pyrex glass and connected to the rest of the apparatus by a glass joint. After having expelled the air from the system by passing a current of carbon dioxide through the apparatus, ⅔ of the fluid was distilled over in a current of CO_2. An aliquot portion of the distillate was then pipetted into a standard flask; into a second flask was placed 0·2 c.c. of a standard diacetyl solution 1: 1,000; both were filled up to 10·0 c.c. with distilled water and after addition of 0·2 c.c. of a freshly prepared 2·5 p.c. solution of o-diamino benzidine hydrochloride and 0·5 c.c. of conc. hydrochloric acid compared in a colorimeter.

For the *determination* of the *acetyl methyl carbinol* 10–12 c.c. of the supernatant fluid, 1·0–1·2 c.c. N. acetic acid and 4·0–5·0 c.c. 20 p.c. solution of ferric chloride were put into the same distilling flask; this mixture was heated for 10 minutes near the boiling point the fractionating column serving as a reflux. Then ⅔ of the volume was slowly distilled over and the total diacetyl (diacetyl + acetoin), thus obtained, determined as above. (Total diacetyl — diacetyl) $\times \frac{88}{86}$ = acetyl methyl carbinol.

The recovery of known amounts of diacetyl by Pien's method is quantitative (Pien, 1937; Cox and Wiley, 1939). Since the oxidation of the carbinol to diacetyl by means of ferric chloride is quantitative and 2, 3-butanediol is not converted into diacetyl in this process (v. Pechmann, Harden and Walpole, 1906; Lemoigne, 1913), one may consider the difference between the amount of diacetyl added to fermenting yeast in nitrogen and of diacetyl plus acetoin recovered as due to

2, 3-butanediol. Nevertheless, I have made sure in one experiment with 10 mg. diacetyl added to a suspension of 0·5 gm. compressed yeast in 1·5 p.c. glucose phosphate solution (gas-space nitrogen; 25° C.; 180 min. fermentation) that 2, 3-butanediol is indeed present among the products at the end of the experiment. Following the method of Lemoigne (1913) in the modification of Kluyver *et al.* (1925) the 2, 3-butanediol was transformed into the characteristic nickel dimethylglyoxime after the removal from the supernatant fluid of diacetyl and acetoin by appropriate distillation.

Because of its specificity the nickel dimethylglyoxime method was used in several experiments to confirm the presence of acetyl methyl carbinol. I found the recommendations of Barnicoat (1935) very useful.

In some cases it was necessary to combine the supernatant fluids of two parallel experiments to provide enough material for the various chemical determinations.

I. PHYTOCHEMICAL REDUCTION UNDER AEROBIC CONDITIONS.

In a previous paper (Gottschalk, 1941) three facts have been established:

1. The oxidation of 1 mol of acetaldehyde to CO_2 and H_2O by compressed yeast is regularly accompanied by the reduction of another mol of acetaldehyde to ethyl alcohol:

$$CH_3CHO + 2O_2 + H_2O + Co \longrightarrow CoH_2 + 2CO_2 + 2H_2O$$

$$CH_3.CHO + CoH_2 \longrightarrow Co + CH_3.CH_2OH \qquad \text{R.Q.} = 1 \qquad [1]$$

2. Acetaldehyde is oxidized by compressed yeast with the same velocity as glucose.

3. The reoxidation of dihydrocoenzyme I (= dihydrocozymase) by acetaldehyde is much quicker than by the flavoprotein-carrier-O_2 system of the yeast cell.

The fact that with acetaldehyde as respiratory substrate 20 p.c. of the oxygen required is provided by an equivalent amount of acetaldehyde acting as H_2-acceptor (equation 1) explains the finding that the oxygen consumption in experiments with acetaldehyde as substrate is only 80 per cent of the value obtained with glucose as substrate. It has been suggested that acetaldehyde is the first hydrogen donator in the oxidative part of yeast respiration according to the following scheme:

$$\begin{aligned}
&\text{triose(phosphate)} + H_2O + Co \longrightarrow CoH_2 + \text{(phospho)glyceric acid}\\
&\text{(phospho)glyceric acid} \longrightarrow H_2O + CH_3.CHO + \underline{CO_2}\\
&CH_3.CHO + \underline{2O_2} + H_2O + Co \longrightarrow CoH_2 + \underline{2CO_2} + 2H_2O\\
&2CoH_2 + \underline{O_2} \longrightarrow 2Co + 2H_2O\\
&\text{triose(phosphate)} + 3O_2 \longrightarrow 3CO_2 + 3H_2O
\end{aligned} \qquad [2]$$

In this scheme two of the three molecules of oxygen consumed oxidize acetaldehyde to CO_2 and H_2O, the third molecule of oxygen reoxidizes the two molecules of reduced cozymase formed intermediately. From the investigations of Euler and Hellström (1938) and Dewan and Green (1938) we know that the reoxidation of the reduced cozymase is effected by a special catalyst referred to as coenzyme factor or diaphorase (flavoprotein dinucleotide). The leucoflavoprotein, thus formed, passes on its hydrogen to suitable carriers, probably cytochromes, which then react with the autoxidizable cytochrome oxidase. In yeast cells the hydrogen transfer by this system proceeds but slowly.

With regard to these findings it seemed possible that in the process of reoxidation of the reduced cozymase (equation 2) an added reducible substance might compete with the slowly reacting flavoprotein-carrier-O_2 system for the hydrogen of the reduced cozymase.

The reducible substance chosen was acetaldehyde because it is physiological to the yeast cell and, in the concentrations used (M/60–M/70), has no inhibitory effect on the respiration. Each experiment was performed with a control, in which another portion of the same yeast suspension was respiring and fermenting in a glucose phosphate solution without added acetaldehyde. The

R.Q. of yeast respiration in glucose solution as well as in acetaldehyde solution being unity, one may assume the same value for the R.Q. if the yeast is respiring in a mixture of glucose and acetaldehyde.

TABLE 1.

O_2 consumption and production of fermentation CO_2, total ethyl alcohol, acetic acid and acetyl methyl carbinol by yeast, respiring and fermenting in a glucose-acetaldehyde-phosphate solution.

200 mg. fresh compressed yeast suspended in M/20 KH_2PO_4 solution. Total volume: 20·0 c.c. 250 mg. glucose puriss. (control) and 250 mg. glucose puriss. + 0·40 c.c. 3·7 p.c. acetaldehyde solution* respectively added.

Temperature: 25° C. Time: 100 min. Gas-space: air.

Range of O_2 tension in mm. Hg. (exclusive of water vapour) from 155–90.

No.	Dry weight of yeast. mg.	Acetaldehyde added. mg.	Acetaldehyde consumed. mg.	Fermentation CO_2 c.c.	Alcohol production. mg.	O_2 uptake c.c.	O_2 uptake p.c. of control.	Acetic acid produced. mg.	Acetyl methyl carbinol produced. mg.
1	57·0	0	0	1·86	3·3	7·77		< 0·1	0
	57·0	14·5	14·5	4·28	15·9	6·17	79·4	1·8	
2	57·0	0	0	1·69	3·1	7·58			
	57·0	14·6	14·6	4·12	15·2	5·96	78·6		0·25
3	60·4	0	0	2·01	4·0	8·02			
	60·4	14·2	14·2	4·41	16·0	6·34	79·1	1·4	
4	57·6	0	0	1·34	2·7 (calculated)	7·91			
	57·6	15·2†	15·2	3·95	16·4	6·39	80·8		0·20
	Acetaldehyde added to compressed yeast as sole substrate without glucose.								
5	56·8	13·6	12·6	5·93 (respiration CO_2)	6·2	5·81	79·5 (compared with glucose as substrate)		

* The exact acetaldehyde content of the solution was determined before each experiment.

† With 15·5 mg. acetaldehyde added traces of acetaldehyde were left at the end of the experiment (Rimini reaction faintly positive).

The results of the experiments recorded in Table 1 demonstrate that the addition of acetaldehyde to compressed yeast, respiring and fermenting in a glucose phosphate solution, decreases the O_2 consumption by 20 p.c., increases the rate of fermentation and besides this effects a further increase in the production of ethyl alcohol. That the O_2 uptake is decreased not by 33⅓ p.c. which might be expected but only by 20 p.c. comes from the fact that part of the acetaldehyde added is oxidized to CO_2 and H_2O analogous to the case with acetaldehyde as the sole substrate (experiment No. 5), thus raising the total O_2 consumption from 66⅔ to 80 p.c. of the control.

The increase in production of alcohol, compared with the control, is due to:

(a) the substitution of one-third of the O_2 consumption of the control by acetaldehyde, which by reoxidizing dihydrocozymase is reduced to ethyl alcohol (equation 3);

(b) the oxidation of part of the added acetaldehyde by 13·33 p.c. of the O_2 consumption of the control yielding 1 mol ethanol per 2 mol oxygen consumed (equation 4);

(c) the dismutation of about 2·4 mg. acetaldehyde to acetic acid and alcohol (equation 5).

$$\text{triose(phosphate)} + H_2O + Co \longrightarrow CoH_2 + \text{(phospho)glyceric acid}$$

$$\text{(phospho)glyceric acid} \longrightarrow H_2O + CH_3\text{·}CHO + \boxed{CO_2}$$

$$CoH_2 + \text{acetaldehyde} \longrightarrow Co + \boxed{\text{alcohol}}$$

$$CH_3\text{·}CHO + \underline{2O_2} + H_2O + Co \longrightarrow CoH_2 + \underline{2CO_2} + 2H_2O \qquad \text{R.Q.} = 1 \qquad [3]$$

$$CoH_2 + \text{acetaldehyde} \longrightarrow Co + \underline{\text{alcohol}}$$

$$\text{triose(phosphate)} + 2O_2 + 2\,\text{acetaldehyde} \longrightarrow 2CO_2 + H_2O + \text{alcohol} + \underline{CO_2 + \text{alcohol}}$$

$$2\,\text{acetaldehyde} + 2O_2 + H_2O \longrightarrow 2CO_2 + 2H_2O + \boxed{\text{alcohol}} \qquad [4]$$

$$2\,\text{acetaldehyde} + H_2O \longrightarrow \text{acetic acid} + \boxed{\text{alcohol}} \qquad [5]$$

In considering the equations [3], [4] and [5] it must be remembered that the acetaldehyde derived from phosphoglyceric acid and the acetaldehyde added are sharing in all the reactions. Since, however, the stoichiometric relations are independent of the acetaldehyde distribution between the various reactions, the above scheme is presented for reasons of clarity.

TABLE 2.

Balance of the acetaldehyde consumption and additional alcohol production by yeast respiring and fermenting in a glucose-acetaldehyde-phosphate solution.

	Increase of alcohol production compared with control.			Total increase in alcohol production.			
	1	2	3	4	5	6	7
No. of Table 1.	Due to a (see text) calculated from O_2-consumption. mg.	Due to b (see text) calculated from O_2-consumption. mg.	Due to c (see text) calculated from acetic acid production. mg.	Calculated (1 + 2 + 3) mg.	Determined. mg.	Acetaldehyde consumption calculated from a, b, c.* mg.	Acetaldehyde disappeared in experiment. mg.
1	10·6	1·1	1·2	12·9	12·6	14·8	14·5
2	10·4	1·0	1·2	12·6	12·1	14·5	14·6
3	11·0	1·1	1·2	13·3	12·0	15·2	14·2
4	10·8	1·1	1·2	13·1	13·7	14·9	15·2

* And added 0·2 mg. taking into account the production of acetyl methyl carbinol (see Table 1).

In Table 2 the acetaldehyde consumption and the increase in alcohol production as calculated from a, b and c are compared with the experimental figures. The close agreement of the experimental with the calculated figures justifies the interpretation that even in the presence of glucose part of the added acetaldehyde is oxidized by compressed yeast and the assumption that this oxidation is effected by the same mechanism and with the same R.Q. as in the experiments with acetaldehyde as the sole substrate (equation 1). The experiments afford definite proof that acetaldehyde added to respiring yeast displaces completely that part of the molecular oxygen which was functioning as a hydrogen acceptor for dihydrocozymase. A comparison of equations [2] and [3] shows that this displacement of 1 mol O_2 by 2 mol acetaldehyde should result in an increased fermentation. The CO_2 in the second reaction of equation [3], deprived of its equivalent O_2 present in the fourth reaction of equation [2], becomes fermentation CO_2 with the alcohol, formed according to reaction 3 in equation [3], as its partner. In all experiments this increase in the rate of fermentation has been observed, the experimental figures being close to the calculated values. As may be seen from equation [3] the expected increase in fermentation CO_2 equals one-third of the oxygen consumed in the control experiment. The experimental and the calculated figures (in brackets) are: 2·42 (2·59), 2·43 (2·53), 2·40 (2·67), 2·61 (2·64).

II. PHYTOCHEMICAL REDUCTION UNDER ANAEROBIC CONDITIONS.

A. Experiments with Diacetyl.

Under strictly anaerobic conditions phytochemical reduction also takes place. The mechanism of the phytochemical reduction under these circumstances was investigated with diacetyl as substrate. From the work of Neuberg and colleagues (Neuberg and Nord, 1919; Neuberg and Kobel, 1925; Nagelschmidt, 1927) it is known that diacetyl added to fermenting yeast undergoes the following reactions:

$$\underset{\text{diacetyl}}{CH_3.CO.CO.CH_3} \xrightarrow{H_2} \underset{\text{acetyl methyl carbinol}}{CH_3.CHOH.CO.CH_3} \xrightarrow{H_2} \underset{\text{2,3-butanediol}}{CH_3.CHOH.CHOH.CH_3} \qquad [6]$$

Both reduction products, acetyl methyl carbinol and 2, 3-butanediol have been isolated and identified.

The experiments in Table 3 demonstrate that diacetyl added to yeast fermenting glucose in N_2 is reduced to acetyl methyl carbinol part of which is further reduced to 2, 3-butanediol, thus confirming the earlier findings of Neuberg and colleagues. *These reductions, too, are effected by the reduced cozymase, and the fermentation acetaldehyde, thus displaced and deprived of its physiological reducing agent, is dismuted to acetic acid and ethyl alcohol.* The figures in Table 3 confirm that acetic acid is produced equivalent to an amount of acetaldehyde displaced by the phytochemically reduced substances. The acetic acid was determined by titration of the

distillate from the cell-free liquid. Though it is extremely improbable that anaerobic fermentation of glucose in the presence of diacetyl yields volatile acids other than acetic acid, I have determined the dissociation constant of the acid in the final distillate. The value obtained was $2{\cdot}28 \times 10^{-5}$ (18° C.), the theoretical value being $1{\cdot}82 \times 10^{-5}$ (18° C.). Acetaldehyde, though it was regularly tested for, could never be found among the products of fermentation.

TABLE 3.

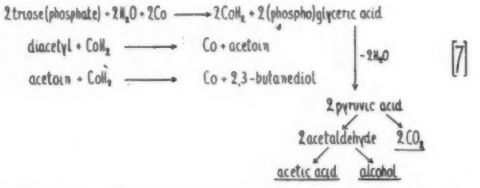
Balance of the phytochemical reduction of diacetyl by fermenting yeast under anaerobic conditions.

300–500 mg. compressed yeast, suspended in M/20 KH_2PO_4 solution. Total volume: 20·0 c.c. In container: 300 mg. glucose (control) and 300 mg. glucose + 0·4 c.c. 2·5 p.c. diacetyl solution respectively. Temperature: 25° C. Time: 2–3 hours. Gas-space: nitrogen (free of oxygen).

		a	b	c	d	e	f	g	h	
Time.	Fresh yeast.	Diacetyl added.	CO_2 produced.	Diacetyl recovered.	Acetyl methyl carbinol left.	Diacetyl reduced to acetyl methyl carbinol a — c.	Acetyl methyl carbinol reduced to 2,3-butanediol (a — e) — (d — 0·3).*	Amount of acetaldehyde displaced by the reactions e and f.	Acetic acid production. Determined.	Calculated from g.
Hrs.	mg.	mg.	c.c.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
2	300	10·0	12·91	3·5	2·2	6·5	4·6	5·7	3·1	3·9
2	500	10·0	22·37	3·7	2·3	6·3	4·3	5·4	not determined	
3	500	10·0	33·56	2·7	2·0	7·3	5·6	6·6	3·8	4·5
3	500	10·0	36·72	2·7	2·0	7·3	5·6	6·6	4·7	4·5
3	500	10·0	35·96	2·5	2·2	7·5	5·6	6·7	4·0	4·6
3	500	10·0	32·63	2·7	1·9	7·3	5·7	6·7	not determined	

* 0·3 is an experimentally determined correction for the amount of acetyl methyl carbinol formed from added acetaldehyde and glucose under analogous conditions. Acetyl methyl carbinol is expressed as diacetyl.

The reduction of diacetyl by fermenting yeast under anaerobic conditions may be formulated in the following way:


2 triose (phosphate) + 2H2O + 2Co → 2CoH2 + 2 (phospho) glyceric acid
diacetyl + CoH2 → Co + acetoin
acetoin + CoH2 → Co + 2,3-butanediol
-2H2O
2 pyruvic acid
2 acetaldehyde
2 CO2
acetic acid
alcohol

[7]

Two consecutive reactions are involved in the phytochemical reduction of diacetyl (equation 8):

$$CH_3.CO.CO.CH_3 \rightleftharpoons CH_3.CHOH.CO.CH_3 \rightleftharpoons CH_3.CHOH.CHOH.CH_3 \qquad [8]$$

In these two stages of reduction one may disregard the tendency for back reaction ($\longleftarrow$); in normal fermentation it is known that the back reaction $CH_3.CHO \longleftarrow CH_3CH_2OH$ is negligible. Further it may be assumed that in the presence of an excess of glucose and within the experimental times the rate of fermentation and consequently the concentration of reduced cozymase and of fermentation acetaldehyde are constant. The initial concentration A_0 of diacetyl and the concentrations A and B of diacetyl and acetyl methyl carbinol respectively after time t of fermentation being experimentally known it was possible to calculate the ratio $R = \frac{k_2}{k_1}$, where k_2 is the rate of reduction of acetyl methyl carbinol and k_1 the rate of reduction of diacetyl.

The reduced cozymase present in the yeast cells is distributed between the three reducible substances, fermentation acetaldehyde, diacetyl and acetoin according to their molecular concentrations and to the affinities of the reducing enzyme system for these substances. The molecular concentrations of diacetyl and of its reduction product acetoin are changing during the experiment. Therefore, if a certain amount of CoH_2, dependent on the result of competition between fermentation acetaldehyde and diacetyl, was available at time t_0 for diacetyl, then at

time t_1 the amount of CoH_2 available for diacetyl and acetoin present is altered by the ratio $\frac{A_{t_1} + RB_{t_1}}{A_0}$, where $A_{t_1} + RB_{t_1}$ is the total amount of reducible substance competing with the fermentation acetaldehyde expressed as diacetyl.

The reduced cozymase diverted from the fermentation acetaldehyde at time t_1 is distributed between the diacetyl and acetyl methyl carbinol present in the ratios $\frac{A_{t_1}}{A_{t_1} + RB_{t_1}}$ and $\frac{RB_{t_1}}{A_{t_1} + RB_{t_1}}$ respectively. For simplicity concentrations are expressed by weight instead of molarity; the volume remains constant during the experiment. The molecular weights of diacetyl and acetyl methyl carbinol being 86 and 88 respectively, B is expressed in terms of A; the correction would be less than the experimental error for the amounts of B in these experiments.

The rate of formation of acetoin $\left(\frac{-dA}{dt}\right)$ cannot be treated as a simple first order mechanism, but must be corrected for the change in the amount of reduced cozymase available to diacetyl. Therefore, the equation becomes $-\frac{dA}{dt} = A \times k_1 \times \frac{A + RB}{A_0} \times \frac{A}{A + RB} = \frac{A^2}{A_0} \times k_1$ [9]

$$\text{thus } A = \frac{A_0}{1 + k_1 t} \qquad [10]$$

The corresponding equation for the rate of formation of 2, 3-butanediol $\left(\frac{dC}{dt}\right)$:

$$\frac{dC}{dt} = B \times k_1 \times R \times \frac{A + RB}{A_0} \times \frac{RB}{A + RB} = \frac{B^2}{A_0} \times k_1 \times R^2 \qquad [11]$$

Now $\frac{dB}{dt} = -\frac{dA}{dt} - \frac{dC}{dt}$

In this equation $\frac{dA}{dt}$ is substituted using equation [10] by $\frac{dA}{dt} = -\frac{k_1 \times A_0}{(1 + k_1 t)^2}$ and using equation [11] one obtains: $\frac{dC}{dt} = \frac{k_1 \times A_0}{(1 + k_1 t)^2} - \frac{dB}{dt} = \frac{B^2}{A_0} \times k_1 \times R^2$

$$\text{or } \frac{dB}{dt} + B^2 \times \frac{k_1 \times R^2}{A_0} = \frac{k_1 \times A_0}{(1 + k_1 t)^2} \qquad [12]$$

The solution of this differential equation is:

$$B = \frac{1}{1 + k_1 t} \times \frac{2 A_0 [(1 + k_1 t)^v - 1]}{(1 + v) + (v - 1)(1 + k_1 t)^v}, \qquad [13]$$

$$\text{where } v = \sqrt{1 + 4R^2}.$$

From [13] is obtained after substituting $(1 + k_1 t)$ by $\frac{A_0}{A}$ using equation [10]

$$\left(\frac{A_0}{A}\right)^v = \frac{1 + a + av}{1 + a - av}, \text{ where } a = \frac{B}{2A}.$$

$$\text{Taking logarithms of both sides, then } v \times \log\left(\frac{A_0}{A}\right) = \log\left(\frac{1 + a + av}{1 + a - av}\right) \qquad [14]$$

In order to solve [14] for v, experimental figures are substituted for A_0, A and B and graphs plotted of $v \times \log\left(\frac{A_0}{A}\right)$ and $\log\left(\frac{1 + a + av}{1 + a - av}\right)$ against v. The solution of v is the intersection of the straight line $v \times \log\left(\frac{A_0}{A}\right)$ with the curve $\log\left(\frac{1 + a + av}{1 + a - av}\right)$.

From $v = \sqrt{1 + 4R^2}$ R may be obtained:

$$R = \tfrac{1}{2}\sqrt{v^2 - 1}.$$

It may be noticed that R is finally obtained without having calculated the individual values of k_1 and k_2 and that the final equation does not contain the time t.

From a large set of experiments the following figures have been taken as the most probable after 3 hours: $A_0 = 10$, $A = 2{\cdot}7$, $B = 1{\cdot}7$. Using these figures $R = 2{\cdot}0$.

This means that the rate of reduction of acetyl methyl carbinol to 2, 3-butanediol is twice the rate of reduction of diacetyl to acetyl methyl carbinol.

B. *Experiments with Acetaldehyde.*

The addition of acetaldehyde to compressed yeast fermenting anaerobically in a glucose phosphate solution results if considered only from the analytical stand-point in the dismutation of an amount of acetaldehyde equal to that added (cf. Table 4).

TABLE 4.

Balance of the acetaldehyde metabolism of yeast fermenting in a glucose-acetaldehyde-phosphate solution under anaerobic conditions.

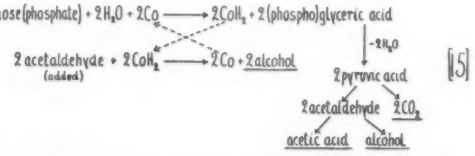
200 or 400 mg. compressed yeast suspended in M/20 KH_2PO_4 solution. Total volume: 20·0 c.c. 60·4 or 250·0 mg. glucose puriss. and 0·28 to 0·37 c.c. 3·7 p.c. acetaldehyde solution added. pH of the suspension at the beginning of the experiment: 4·3, at the end of the experiment: 3·2. Gas-space: nitrogen (free of oxygen). Temperature: 25° C. Time: 100–125 min.

	a	b	c	d	e	f	g	h	i	k	l	m
			Acetaldehyde		Fermentation			Extra alcohol produced.		Acetic acid produced.		
Time.	Amount of fresh yeast.	Glucose added.	added.	consumed.	CO_2	Alcohol (calculated from e)	Total alcohol produced (determined).	determined (g — f).	calculated.*	determined.	calculated.*	Acetyl methyl carbinol produced.
min.	mg.	mg.	mg.	mg.	c.c.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
100	200	250	0	0	14·03							
100	200	250	10·2	10·2	8·44	17·3	22·3	5·0	5·2	6·0	6·8	0·3
100	200	250	11·4	10·6	8·02	16·3	16·1	5·8	5·4	6·6	7·0	0·3
105	400	60·4†	11·0	11·0	11·67	24·0	28·9	4·9	5·6	6·4	7·3	not determined
125	400	60·4†	13·5	13·5	11·86	24·4	30·8	6·4	6·9	8·1	9·0	not determined

* The calculated figures for dismuted alcohol and dismuted acetic acid are from the equations extra alcohol $= \frac{d - m}{2} \times \frac{46}{44}$ and acetic acid $= \frac{d - m}{2} \times \frac{60}{44}$.

† Fermented to the complete consumption of the glucose added as determined in the fermented liquid after the experiment by the Hagedorn-Jensen method.

In this case, just as in the case of aerobic phytochemical reduction of acetaldehyde, the fermentation acetaldehyde and the added acetaldehyde will share in both reactions, in the dismutation and in the reoxidation of dihydrocozymase. Accordingly the reactions between the acetaldehyde molecules of different origin and the enzyme systems concerned will in part follow the scheme [15] and in part these molecules will exchange places in the scheme.



There is a remarkable difference between the rate of dismutation when acetaldehyde is added as sole substrate to compressed yeast and that when it is added to fermenting yeast. As formerly shown (Gottschalk, 1941) 200 mg. compressed yeast dismute in 2 hours at 25° C. 2·4–2·7 mg. acetaldehyde under anaerobic conditions. Fermenting yeast, under otherwise the same conditions, dismutes 10·2–10·6 mg. acetaldehyde. In this connection reference may be made to the interesting findings of Nilsson (1930) obtained with dried yeast in the presence of NaF.

The rate of anaerobic fermentation is considerably inhibited by the addition of acetaldehyde. In Table 5A is shown the inhibitory effect of two different acetaldehyde concentrations on the fermentation of an excess of glucose. The figures demonstrate that the inhibitory influence decreases gradually; after a certain time, dependent on the acetaldehyde concentration, the rate of fermentation is the same as in the control experiment if stages are compared in which equal glucose concentrations are left (as calculated from the CO_2 production). For instance, in experiment 2 (Table 5A) the volume of CO_2 in the control after 90 minutes amounts to 19·91 c.c., after 120 minutes to 25·71 c.c., in the acetaldehyde experiment after 120 minutes to 19·60 c.c. and after 150 minutes to 25·66 c.c. No acetaldehyde was left at the end of the experiments.

TABLE 5.

The degree of inhibition of alcoholic fermentation in N_2 by various concentrations of acetaldehyde.

A. Fermentation in the presence of an excess of glucose.

300 mg. compressed baker's yeast, suspended in M/20 KH_2PO_4 solution. Total volume: 20·0 c.c. In container: 300 or 400 mg. glucose (control) and 300 or 400 mg. glucose + 0·2 to 0·3 c.c. 3·5 p.c. acetaldehyde solution respectively. Temperature: 25° C. Time: 2–2½ hrs. Gas-space: nitrogen (free of oxygen).

	Amount of fresh yeast. mg.	Glucose added. mg.	Acetaldehyde added. mg.	CO_2 at N.T.P. produced after 30 min. c.c.	60 min. c.c.	90 min. c.c.	120 min. c.c.	150 min. c.c.	Remarks
1	300	300	0	6·29	13·53	20·13	26·21		
	300	300	7·0	4·26	10·35	17·10	23·01		no acetaldehyde left
2	300	400	0	6·06	13·21	19·91	25·71	31·25	
	300	400	10·5	3·31	7·66	13·39	19·60	25·66	no acetaldehyde left

The experiments of Table 5B, carried out with smaller amounts of glucose and various concentrations of acetaldehyde, were continued until the sugar had completely disappeared. These results, too, demonstrate the gradually diminishing inhibitory effect of acetaldehyde upon fermentation; furthermore, they illustrate the fact that the final balance of glucose utilization is not affected by the presence of acetaldehyde. Both with glucose and with glucose plus acetaldehyde as substrates, compressed yeast ferments only 77 p.c. of the glucose to CO_2 and alcohol, the rest being assimilated.

TABLE 5.

B. Complete fermentation of the glucose added.

400 mg. compressed yeast, suspended in M/20 KH_2PO_4 solution. Total volume: 20·0 c.c. In container: 60·3 or 61·7 mg. glucose puriss. (control) and 60·1–85·0 mg. glucose puriss. + 0·4 to 0·7 c.c. 2·8 p.c. acetaldehyde solution* respectively. Temperature: 25° C. Gas-space: nitrogen (free of oxygen).

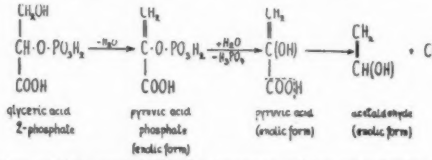
Amount of fresh yeast.	Glucose added.	Acetaldehyde added.	CO_2 at N.T.P. produced after 30 min.	45 min.	60 min.	90 min.	100 min.	105 min.	120 min.	150 min.	160 min.	180 min.	195 min.	Glucose fermented / Glucose consumed.	Remarks.
mg.	mg.	mg.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.	p.c.	
400	60·3	0	7·42	10·40	11·46	—	—	—	—	—	—	—	—	76·4	†
400	60·4	11·0	2·89	—	6·31	10·79	—	11·67	—	—	—	—	—	77·7	‡
400	74·0	17·5	2·26	—	4·70	7·67	—	—	10·98	13·61	13·96	—	—	75·8	§
400	85·0	20·2	2·20	—	4·14	6·35	—	—	8·91	12·19	—	15·73	16·44	77·8	§
400 brewery bottom yeast	61·7	0	4·61	6·52	8·54	10·82	—	12·20	—	—	—	—	—	79·5	†
400 brewery bottom yeast	60·1	13·5	5·74	—	9·99	12·89	13·15	—	—	—	—	—	—	87·9	‖

* The exact acetaldehyde content of the solution was determined before each experiment.

Remarks. † No glucose left. ‡ No glucose left; no acetaldehyde left. § No glucose left; no acetaldehyde left. Traces of nickel dimethylglyoxime (<1 mg.) obtained. No 2, 3-butanediol detectable. ‖ No glucose left; no acetaldehyde left. Traces of nickel dimethylglyoxime (about 1·5 mg.) obtained.

The inhibition of fermentation by acetaldehyde in the concentrations applied is seen to be completely reversible. The gradual transition from the inhibited to the normal rate of fermentation indicates that the metabolism of the acetaldehyde added interferes with some part of the fermenting system and that this interference ceases after the consumption of the acetaldehyde. Such an interference may occur at two places. Firstly: the dismutation of acetaldehyde to acetic acid and ethanol is a cozymase-linked reaction; therefore part of the cozymase is diverted from

the triosephosphoric enzyme to the aldehyde mutase. Secondly: evidence from acyloin synthesis, effected by fermenting yeast plus acetaldehyde (Neuberg and Hirsch, 1923), has led to the suggestion that the fermentation acetaldehyde may be in a more reactive form than is the added acetaldehyde perhaps being formed in the following reactions:



[15a]

The less reactive added acetaldehyde because of its relatively high molecular concentration would displace some of the fermentation acetaldehyde in the reaction $CH_3CHO + CoH_2 \longrightarrow Co + C_2H_5OH$; this displacement would result in a slower reoxidation of the dihydrocozymase. Both factors will bring about a diminution in the amount of cozymase available to the apozymase thus inhibiting fermentation. Such an interpretation is supported by experiments with brewery bottom yeast which according to Neuberg and Gottschalk (1925) contains much more cozymase than baker's yeast. In this case the addition of acetaldehyde not only does not inhibit the rate of fermentation but exercises a marked stimulatory effect (Table 5B). Such an effect is known to be exerted by many aldehydes and other substances acting as hydrogen acceptors. The lack of inhibition by acetaldehyde on fermentation with bottom yeast discounts the suggestion that the results with compressed yeast are due to a specific inhibition of carboxylase by the added acetaldehyde.

Recently Lennerstrand (1941) has published evidence that apozymase contains enzymes which act destructively on cozymase and that cozymase can be partly reactivated by addition of a mixture of glucose, hexose-diphosphoric acid and PO_4 ions. It may well be that the above mentioned difference in the rate of acetaldehyde dismutation by compressed yeast alone and by fermenting yeast is due to this mechanism.

The production of acetyl methyl carbinol by fermenting yeast in the presence of less than 0·1 p.c. acetaldehyde concentration (cf. Table 4 and 5B) is very small. Already Neuberg and Reinfurth (1923) have observed that compressed top yeast is an unsuitable yeast race for the demonstration of the biochemical acyloin synthesis. Recently Dirscherl and Schöllig (1938) have reported that with acetaldehyde concentrations as low as 1 p.c. the yield of acetoin by fermenting yeast amounts only to 7·5 p.c. of the theory, a value probably still further diminishing with lower concentrations of acetaldehyde.

DISCUSSION.

From the experiments described in the previous paper it has been concluded that in the series of reactions by which the complete oxidation of 1 mol of triosephosphate to CO_2 and H_2O is effected by compressed yeast two reactions are coenzyme-linked: the oxidation of triosephosphate to phosphoglyceric acid and one step in the final oxidation of acetaldehyde. The experiments described in the first part of this paper confirm this conclusion. Acetaldehyde added to compressed yeast respiring and fermenting in a glucose plus phosphate solution is reduced to ethyl alcohol in an amount explainable only if in normal yeast respiration (in glucose) 2 mol of dihydrocozymase are intermediately formed per mol of triosephosphate oxidized to CO_2 and H_2O. These 2 mol CoH_2 are reoxidized by the reduction either of 1 mol O_2 (normal respiration with glucose) or of 2 mol acetaldehyde (respiration in the presence of glucose plus acetaldehyde).

In the same paper, the slow process of regeneration of CoH_2 by molecular oxygen as compared with the extremely quick reaction $CH_3.CHO + CoH_2 \longrightarrow Co + C_2H_5OH$ has been made responsible for the inhibitory effect of respiration on the process of alcoholic fermentation and of total carbohydrate consumption by yeast cells (Pasteur effect). If this theory is correct, one might perhaps expect that by addition of acetaldehyde, in concentration sufficient to displace that part of the oxygen consumed which in normal respiration reoxidizes the dihydrocozymase, the rate of fermentation should increase, even though the added acetaldehyde may not equal the fermentation acetaldehyde in its reactivity with dihydrocozymase. This is not the case. The small increase in fermentation observed is due not to any increase in the rate of decarboxylation but to the fact that the oxygen consumed in the reoxidation of CoH_2 is displaced by added

acetaldehyde; consequently the corresponding carbon dioxide appears under the heading of fermentation CO_2.

The experiments recorded in Tables 4 and 5 explain why the Pasteur effect is not cancelled by the addition of acetaldehyde to yeast respiring in glucose solution. M/80 acetaldehyde, added to fermenting yeast in nitrogen, reduces the CO_2 production to about 60 p.c. of the control value (100 min.). This inhibition is mainly due to a diversion of part of the cozymase from the triosephosphoric enzyme to the aldehyde mutase; perhaps the displacement of some of the supposed reactive form of acetaldehyde by the more stable added acetaldehyde in the reaction with CoH_2 adds to the effect. One must conclude from this that one factor controlling the distribution of the cozymase between the triosephosphoric enzyme and the aldehyde mutase is the ratio of the molecular concentrations of their specific substrates triosephosphate and acetaldehyde. If in yeast cells, respiring and fermenting in a glucose-acetaldehyde mixture, more regenerated cozymase becomes available because the dihydrocozymase is reoxidized by the added acetaldehyde much faster than by molecular oxygen, then part of this surplus of cozymase is seized by the aldehyde mutase; another part is diverted to the enzyme system which oxidizes acetaldehyde to CO_2 and H_2O. The result is the experimentally observed dismutation and oxidation of portion of the added acetaldehyde. Thus a real increase in fermentation is prevented.

The inhibition of alcoholic fermentation and of total carbohydrate consumption in nitrogen by the addition of acetaldehyde can be considered as a counterpart to the Pasteur effect in yeast cells. In both cases the inhibition is reversible and is caused by a deficiency of cozymase available for the triosephosphoric enzyme. In normal yeast respiration (in glucose) this lack of cozymase is mainly caused by the slow reoxidation of dihydrocozymase (cf. above). Possibly the fact that the oxidation of 1 mol of glucose requires twice as much cozymase as the fermentation of 1 mol glucose may also contribute to the shrinkage of cozymase available for the completion of the triosephosphoric enzyme.

The relationship between the Pasteur effect and the phytochemical reduction under aerobic conditions is evident. *The sluggish reduction of molecular oxygen by dihydrocozymase mediated through the flavoprotein-carrier system offers any other reducible substance the opportunity to compete with molecular O_2 for the hydrogen of CoH_2. The result of this competition depends on the affinity of the reducing enzyme of fermentation for the reducible substance added,* but is independent of the latter's molar concentration provided that, which experimentally holds, enough reducible substance is present to saturate its enzyme.

General equation of the phytochemical reduction under aerobic conditions.

$$\begin{aligned}
&\text{triose(phosphate)} + H_2O + Co \longrightarrow CoH_2 + \text{(phospho)glyceric acid}\\
&\text{(phospho)glyceric acid} \longrightarrow H_2O + CO_2 + CH_3.CHO\\
&CH_3.CHO + 2O_2 + H_2O + Co \longrightarrow CoH_2 + 2CO_2 + 2H_2O\\
&2CoH_2 + 2\ -\overset{\overset{O}{\|}}{C}-H(\text{or } R) \longrightarrow 2Co + 2\ -\overset{\overset{H}{|}}{\underset{\underset{OH}{|}}{C}}-H(\text{or } R)
\end{aligned} \qquad [16]$$

General equation of the phytochemical reduction under anaerobic conditions.

$$\begin{aligned}
&2\,\text{triose(phosphate)} + 2H_2O + 2Co \longrightarrow 2CoH_2 + 2\,\text{(phospho)glyceric acid}\\
&2\,\text{(phospho)glyceric acid} \longrightarrow 2H_2O + 2CO_2 + 2CH_3.CHO\\
&2CH_3.CHO + H_2O \longrightarrow CH_3.COOH + C_2H_5OH\\
&2CoH_2 + 2\ -\overset{\overset{O}{\|}}{C}-H(\text{or } R) \longrightarrow 2Co + 2\ -\overset{\overset{H}{|}}{\underset{\underset{OH}{|}}{C}}-H(\text{or } R)
\end{aligned} \qquad [17]$$

The dependence of the rate of phytochemical reduction on the rate of fermentation has not yet been investigated. My own experiments in this regard justify only the conclusion that the rate of phytochemical reductions is not directly proportional to the rate of fermentation. Even at the very low rate of fermentation observed in the presence of oxygen some of the phytochemical reduction is probably effected by the anaerobic mechanism.

With regard to the physiological aspect of the phytochemical reductions it may be concluded that under anaerobic conditions there would be scarcely any measurable phytochemical reduction if the concentration of the reducible substance present were of the order of that of the fermentation acetaldehyde because of the high affinity of the reducing enzyme of fermentation for acetaldehyde. One molecule of the enzyme (assuming a molecular weight of 70,000) catalyses in the presence of excess acetaldehyde the oxidation of 28,500 molecules of dihydrocoenzyme I per min. at 20° C. (Negelein and Wulff, 1937). The phytochemical reduction of an aldehyde or ketone added under anaerobic conditions is a reaction enforced by the relatively high concentration of the substrate added. In the presence of oxygen the conditions are quite different. Here an organic hydrogen acceptor, even in very low concentration, has a good chance for reduction, because it has to compete only with the slowly acting flavoprotein-carrier-O_2 system. It may well be that in the presence of oxygen under certain intracellular conditions intermediates formed in the breakdown of foodstuffs and containing an aldehyde or a keto group or a double bond between C-atoms temporarily function as hydrogen acceptors for the reduced cozymase. The well-known fact that aeration increases considerably the production of glycerol in the process of alcoholic fermentation by living yeast cells is an example of this type of reaction. It seems worth while to reconsider from this aspect the mechanism of the production of fatty acids from carbohydrates split products. This process consists essentially of aldol condensations and hydrogenations of double bonds between C-atoms.

SUMMARY.

Acetaldehyde added to compressed yeast respiring and fermenting in glucose phosphate solution displaces completely that part of the oxygen consumed which in normal respiration (with glucose as sole substrate) reoxidizes dihydrocozymase, a reaction whose slowness is mainly responsible for the Pasteur effect. By displacing molecular oxygen as hydrogen acceptor for CoH_2 the aldehyde is phytochemically reduced. The amount of this reduction is explainable only if per mol of triosephosphate completely oxidized 2 mol CoH_2 are intermediately produced; this confirms an earlier conclusion that the oxidation of triosephosphate to CO_2 and H_2O by baker's yeast involves coenzyme I linked reactions.

Further, a quantitative study has been made of the two steps involved in the phytochemical reduction of diacetyl by fermenting yeast under anaerobic conditions (diacetyl $\longrightarrow$ acetyl methyl carbinol $\longrightarrow$ 2, 3-butanediol). The fermentation acetaldehyde, deprived by these reductions of its CoH_2, is dismuted to acetic acid and ethanol. The rate of reduction of acetyl methyl carbinol to 2, 3-butanediol is twice the rate of reduction of diacetyl to acetyl methyl carbinol.

Acetaldehyde (M/80 concentration) added to fermenting baker's yeast in nitrogen inhibits reversibly the rate of fermentation mainly because part of the cozymase is diverted from the triosephosphoric enzyme to the aldehyde mutase.

The physiological significance is discussed of the mechanism by which in the process of respiration phytochemical reductions are effected.

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THE BREAKDOWN OF COMMERCIAL STARCHES BY MUSCLE EXTRACT

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Fresh muscle extracts, poisoned by iodoacetic acid, can be used for the preparation of either glucose-1-phosphate or the "equilibrium ester" (Kiessling, 1938). As shown previously, it is convenient to use starch as substrate (Fantl and Anderson, 1941). With most commercial starches no difficulties have been encountered in obtaining good yields of glucose-1-phosphate, but, with certain samples of wheat starch, we failed to isolate hexose-6-phosphate in the expected amounts.

An analytical study gave results recorded in Table 1.

TABLE 1.

Breakdown of starches by rabbit muscle extract.

Reaction Mixture: 100 ml. extract (Kiessling) containing 0·14 p.c. iodoacetic acid, 0·66 p.c. starch, M/5 PO_4, pH = 6·50 (glass electrode). Incubated in an atmosphere of N_2 — 5 p.c. CO_2 at 23° C.

	After 15 mins. incubation.		After 45 mins. incubation.	
Starch used.	Glucose–1–PO_4.	Hexose–6–PO_4.	Glucose–1–PO_4.	Hexose–6–PO_4.
Maize	41·6	19·9	30·7	42·7
Potato	37·8	20·6	29·0	48·2
Rice	34·8	24·4	29·0	44·0
Wheat	44·8	10·2	47·0	17·7

The figures are given in mg. P. per 100 ml. reaction mixture. The estimations were carried out in trichloracetic acid filtrate after removal of inorganic phosphate by magnesia mixture. Glucose–1–phosphate was estimated by 10 mins. hydrolysis in N.H_2SO_4 at 100° C. and hexose–6–phosphate by 10 mins. hydrolysis in N.KOH. In addition to the phosphorus estimations, the barium salts of the reaction products were prepared. The yields were approximately the same in all cases. Here the hexose component was also estimated and the results were similar to those shown in Table 1.

Since all starches used showed a total phosphorus uptake not differing by more than 10 p.c. it is evident that the first step of the degradation yielding glucose–1–phosphate was not inhibited. However, the ratio of 1 to 6 ester was significantly changed in the case of wheat starch which suggested that this starch either contained an inhibitor of phosphoglucomutase or lacked an accelerator which was present in the other starches.

From the results of Cori *et al.* (1938), Lehmann (1939), and Gill and Lehmann (1939) it is known that metal ions have either an accelerating (Mg, Mn, Ni, Co) or inhibiting (Cu, Fe, Zn) influence on phosphoglucomutase. Therefore, ashes of the starches were prepared and their solutions added to the reaction mixtures.

TABLE 2.

Influence of ashes from commercial starches on hexose–phosphate formation.

Conditions of experiments similar to those given in Table 1. Incubation time 45 mins., pH = 6·45, temp. 25° C. Ash prepared from an amount of starch equal to that taken for each experiment.

Starch used.	Addition.	Glucose–1–phosphate.	Hexose–6–phosphate.
Potato	nil	30·7	29·5
Potato	Ash from wheat	52·1	16·2
Wheat	nil	52·3	11·7
Wheat	Ash from potato	52·9	14·6

The values are given in mg. P. per 100 ml. reaction mixture. The figures recorded in Table 2 indicate that 50 p.c. of the hexose–phosphates formed from the potato starch was converted into hexose–6–phosphate and that the ash solution of the wheat starch exerted an inhibitory influence on this conversion. The potato ash, added to the wheat starch, had no accelerating influence on the formation of hexose–6–phosphate.

[1] Working under a full time grant from the National Health and Medical Research Council.

The same ratios were obtained by estimating the sugar components in the isolated barium salts using the reducing power before and after 10 mins. hydrolysis in $N.H_2SO_4$.

Analyses of the starch ashes are shown in Table 3.

TABLE 3.

Starch.	Ash content in p.c.	Spectrographic analysis*										Zinc estimated by dithizone†
		Ca	Zn	Cu	Mg	Mn	P	Si	B	Fe	Pb	mg. p.c.
Wheat	0·16	2	10	tr.	4	tr.	tr.	A	A	A	A	3·95
Potato	0·27	4	A	tr.	3	A	tr.	tr.	A	tr.	A	0·8
Rice	0·7	10	A	tr.	10	10	10	10	10	tr.	v. f. tr.	—
Maize	0·12	5	A	v. f. tr.	5	A	tr.	A	tr.	A	A	—

A signifies absent in spectrum; tr. signifies trace only; v.f.tr. signifies very faint trace. A value of 10 has been assigned to the sample in which the element occurred in the greatest amount, and the other samples graded accordingly.

* We are indebted to Mr. Keith Winsor of the E.M.F. Electric Co. Pty. Ltd. for these analyses and evaluation of the data.

† Method used for zinc estimation described by Sylvester and Hughes (1936), Walkley (1942) and O'Brien (1942).

These analyses suggest that the zinc content of the wheat starch was responsible for the inhibition of the phosphoglucomutase. Commercially-prepared starches vary considerably in their ash content and composition. Therefore, an adverse influence on the formation of either glucose–1– or hexose–6–phosphate may be expected. However, it is still possible to use such starches without further purification by making use of the specific influence of different metal salts as illustrated in Table 4.

TABLE 4.

Reaction mixture of similar composition to Table 1, Temp. 27° C.

Starch.	After 15 mins. incubation.		After 45 mins. incubation.	
	Glucose–1–PO_4.	Hexose–6–PO_4.	Glucose–1–PO_4.	Hexose–6–PO_4.
Potato	53·2	9·2	52·1	22
Potato + 2×10^{-5} M. Zn SO_4	62·2	6·5	65·4	11·1
Wheat	61	6·8	69	11·5
Wheat + 2×10^{-5} M. Mn SO_4	28·8	22·7	20·8	38

These results indicate that an addition of 2×10^{-5} M. Zn is of advantage in the preparation of glucose–1–phosphate, whereas, in the case of the preparation of hexose–6–phosphate, an addition of manganese is advisable to abolish any inhibition by zinc.

SUMMARY.

Certain commercial samples of starches were found to contain small amounts of zinc which were sufficient to inhibit, to a large extent, the formation of hexose-6-phosphate by rabbit muscle extract. Addition of manganese abolishes this inhibition.

Acknowledgment. We are indebted to Mr. J. F. Nelson for carrying out the phosphorus estimations.

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SOME OBSERVATIONS ON FASCICULATION AND FIBRILLATION IN SKELETAL MUSCLE WITH SPECIAL REFERENCE TO THE ACTION OF NOVOCAIN ON THE NEUROMUSCULAR MECHANISM

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The well-known action of physostigmine in producing fasciculation of mammalian skeletal muscle is not readily explained in terms of its inhibiting action on the esterase which inactivates acetylcholine. One difficulty is that after the nerve has degenerated fasciculation can no longer be obtained in the denervated muscle, although this has become extremely sensitive to acetylcholine. Moreover, Masland and Wigton (1940) have recently observed antidromic impulses in the anterior roots during fasciculation produced by an allied drug, prostigmine.

In a different category from this coarse fasciculation is the fine fibrillation of denervated muscle which is influenced only slightly, if at all, by physostigmine (Langley and Kato, 1915) (Rosenblueth and Luco, 1937). Denny-Brown and Pennybacker (1938) expressed the view that this spontaneous activity is due to the action of acetylcholine on the greatly sensitized muscle. Against this view, however, is the resistance of fibrillation to the action of curare which reduces the response of denervated muscle (though not so definitely as that of innervated muscle) to acetylcholine.

Langley and Kato (1915) showed in the cat and rabbit that physostigmine ceased to produce fasciculation 2 days after denervation, and fibrillation appeared 5 days after denervation. In no single series of experiments has there been a correlated study of the time relationship of the loss of fasciculation, and the later onset of fibrillation with the development of the change in sensitivity to acetylcholine. Indeed the time of onset of the change in sensitiveness to acetylcholine has not been clearly determined for either cat or rabbit. Brown (1937) stated that one cat had only twice the normal sensitivity to acetylcholine 4 days after denervation and the cats of Rosenblueth and Luco (1937) were not examined until 6 days after denervation at which time hyper-sensitivity was already established. Lissak, Dempsey and Rosenblueth (1939) state that a contractural response was found in a muscle of the cat 3 days after denervation, but no indication is given of the degree of sensitivity relative to that of normal muscle.

This paper is concerned with the time relationships of the disappearance of fasciculation, the loss of indirect excitability, the onset of fibrillation and sensitization to acetylcholine. In addition, the actions of curare on fibrillation and on the acetylcholine sensitivity of denervated muscle were observed simultaneously in one and the same animal.

Because some of the facts outlined above suggested that fasciculation depends on an intact motor nerve, an attempt was made to bring about "paralysis" of all the nerve endings in a given region by intra-arterial injection of novocain. This method, however, led to no conclusion about the site of action of physostigmine because it was found that novocain had a curare-like action abolishing fasciculation much more readily than indirect excitability. Previous observers, Fulton (1921), Bremer and Titeca (1930), Riesser and Neuschloss (1921) and more recently Harvey (1939) and Macgregor (1939) had observed a curare-like action of novocain but they did not describe its action on fibrillation or fasciculation. In this paper are recorded further observations on the action of novocain on the neuromuscular mechanism.

[1] This work was carried out by means of a grant from the National Health and Medical Research Council.

METHODS.

Observations were made on forty cats. Some of these animals were operated upon with aseptic precautions and a length of 1 cm. removed from the left sciatic nerve from 40 hours up to several weeks before the final experiment. For the final experiment, the animal was anaesthetized with ether and decapitated, although in some experiments chloralose (10 c.c. of 1 p.c. per kg.) was given intravenously. An intravenous injection of 1–2 mg. of atropin was given. The left sciatic nerve, if still intact, was then divided and drawn on to platinum electrodes for stimulation and the gastrocnemius muscle was exposed. Intra-arterial injections were made into the cut stump of a muscular branch of the femoral artery distal to the origin of the deep femoral artery. No attempt was made to restrict the circulation to the gastrocnemius-soleus group. The circulation to the limb was occluded during acetylcholine injections and for novocain injections this was maintained for 30–60 seconds. Injections were given in Ringer's solution in a volume of 1 c.c. To record the responses of the gastrocnemius the lower ends of the femur and the tibia and fibula were supported by Palmer's thermometer clamps attached to upright rods fixed to the operating table. The tendon of Achilles was attached to a recording lever usually of isometric type; but to record fasciculation an isotonic lever was employed. Fibrillation was observed with the naked eye. Stimulation of the nerve or muscle was carried out by single break shocks from an induction coil. Rapidly repeated stimuli at 60–100 per second were also obtained from an induction coil with a variable rate interrupter. For direct stimulation of the muscle, electrodes were placed at the lower and upper ends of the muscle.

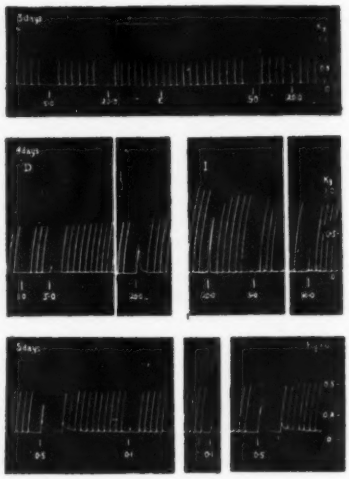

Fig. 1. Isometric records of gastrocnemius muscles from 3 spinal cats, 72, 96 and 120 hours after denervation. In the middle tracing the responses of the corresponding innervated gastrocnemius are shown on the right. The doses of acetylcholine are indicated by the figures which represent γ. At E in the upper figure and between the first two panels of the bottom figure 1 mg. of physostigmine sulphate was injected intravenously. The unlettered responses are to maximal break stimuli. Time in half minutes.

RESULTS.

(1) *Changes within the First Week of Denervation.*

Magnus (1908) stated that the twitching of skeletal muscle following the intravenous injection of physostigmine was unobtainable after 18–27 days of denervation, whereas Langley and Kato (1915) showed that it was unobtainable after 2 days, i.e. when indirect excitability had ceased. My results are in entire agreement with those of Langley and Kato. In addition it was shown that the change in response to acetylcholine followed a completely different time course.

Using the technique of "adjacent" intra-arterial injection described above, acetylcholine in suitable dosage regularly produced a single twitch in the innervated muscle. The threshold dose was about 20γ. After eserinization 20γ frequently gave a response as large or nearly as large as that of a maximal nerve volley and 5 to 10γ produced a threshold response. In muscle after a week or more of denervation the threshold dose was usually less than 0·1γ, the muscle responding with both a twitch and a contracture. Eserine made little difference to the sensitivity but, as Brown (1937) observed, it enhanced the contracture (both in height and duration) rather than the twitch (see Fig. 1). The somewhat larger size of these doses than those used by Brown, Dale and Feldberg (1936) and by Brown (1937) is attributable to the longer range of injection and the larger volume (and therefore greater dilution) of the injected drug. However, responses were regular and uniform.

If the recently denervated muscle gave no response to 10γ or 20γ of acetylcholine it was considered insensitive. When there was a doubtful or slight increase in sensitivity, e.g. if it responded to 5γ, its response was compared with that of its innervated fellow from the other limb. Table 1 illustrates the results. From approximately 2 days onwards when the nerve ceased to conduct a single stimulus the intravenous injection of doses up to 1 to 2 mg. of physostigmine per kg. produced no fasciculation in the denervated muscle. During and at the end of the third day the high degree of sensitivity to acetylcholine was not present and at the end of 4 days the muscle had no more than twice the sensitivity of its normal fellow. Not until the end of the fifth day was the high degree of sensitivity established. At this stage fibrillation first appeared. The presence of the contractural element of the acetylcholine response could however be seen in

animals 64, 72 and 96 hours after denervation. In the upper tracing of Fig. 1 the response of a gastrocnemius muscle 72 hours after denervation is shown both before and after eserinization. This muscle responded with a twitch only. The middle tracing shows the responses of the normal and denervated muscles of an eserinized animal 96 hours after denervation. The contractural element in the muscle response can be clearly seen and the sensitivity is about twice that of the normal side. The lower tracing illustrates the high degree of sensitivity 120 hours after denervation. It also shows how eserine enhances the magnitude of the contracture rather than the twitch.

TABLE 1.

Period of Denervation.	Indirect Stimulation.	Eserine Fasciculation.	Acetylcholine responses. Denervated.	Innervated.	Fibrillation.
40 hours	Present	Present	—	—	Absent
48 hours	Present	Feebly present	50γ Twitch	—	Absent
64 hours	Absent for single stimuli	Absent	20γ Nil		
	Feeble response with repeated stimuli		50γ Twitch and contracture	—	Absent
72 hours	Absent	Absent	5γ Nil 20γ Twitch only	—	Absent
72 hours	Absent	Absent	20γ Nil 50γ Twitch and contracture	—	Absent
96 hours	Absent	Absent	* 1γ Nil 5γ Twitch and contracture	* 5γ Nil 10γ Twitch	Absent
96 hours	Absent	Absent	1γ Nil 5γ Contracture just perceptible	5γ Nil 10γ Twitch	Absent
120 hours	Absent	Absent	0·1γ Twitch and contracture	—	Present
144 hours	Absent	Absent	0·1γ Nil 0·5γ Twitch and contracture	—	Present

* Animals previously injected with physostigmine.

(2) *The Effect of Curare on the Fibrillation and on the Acetylcholine Response in the same Denervated Muscle.*

The absence of effect of curare on denervation fibrillation was amply confirmed in many experiments. In some experiments in which the acetylcholine response of denervated muscle was examined before and after the administration of curare it was found that doses which were without effect on fibrillation considerably reduced the sensitivity to acetylcholine. Thus in one experiment an injection of curare which abolished indirect excitability of the innervated gastrocnemius had no visible effect on the fibrillation of the denervated gastrocnemius. In the same animal 0·1γ of acetylcholine before curare gave a good response, after curare the threshold dose was 0·5γ, and 5γ produced an effect comparable with the original response to 0·1γ. In another experiment 0·5γ of acetylcholine gave a good response in the denervated gastrocnemius. After the injection intravenously of twice the dose of curare necessary to abolish indirect excitability of the innervated muscle, the threshold dose of acetylcholine was greater than 50γ, and 100γ produced a response equal in magnitude to the original 0·5γ. In this experiment fibrillation though reduced in intensity was still present.

(3) *The Effect of Intra-arterial Injections of Novocain on Innervated Muscle.*

(a) *Fasciculation.* The injection of 0·5–2·0 mg. per kg. of physostigmine produced coarse twitching of the musculature of the body including the gastrocnemius whose nerve supply had been severed at the beginning of the experiment. The intra-arterial injection of suitable doses of novocain in volumes of 1 c.c. abolished the fasciculation in the gastrocnemius but had little effect on the response to a maximal nerve volley. Larger doses reduced the latter response but generally speaking the dose necessary to abolish indirect stimulation completely was about 50 times the size of the minimal dose necessary to abolish fasciculation. Injections of 1 c.c. of normal saline with occluded circulation for 30 to 60 seconds were without effect on fasciculation or on the response of the muscle to indirect stimulation or to acetylcholine. Fig. 2 shows the isotonic record of the gastrocnemius of an atropinized cat which had been injected intravenously with 1·5 mg. of

physostigmine per kg. Injections of 2, 10 and 20 mg. of novocain were given at the marks. A dose of 2 mg. almost completely abolished the fasciculation for 45 secs. when it returned to half its original amplitude. A dose of 10 mg. abolished the fasciculation except for an occasional twitch. A dose of 50 mg. given in the interval between the panels only reduced the response to a maximal nerve volley to the degree shown and a further 100 mg. was required to abolish neuromuscular conduction. Frequently 2 mg. of novocain completely abolished fasciculation for the duration of an experiment (up to half an hour). A dose of this magnitude had little or no effect on the response to a maximal nerve volley.

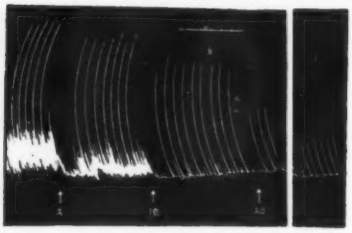

Fig. 2. Isotonic record of innervated gastrocnemius muscle of a spinal cat. At the arrows, 2, 10 and 20 mg. of novocain were injected intra-arterially. Between the two panels, 50 mg. of novocain were given. The unlettered responses are to maximal break shocks. Time in half minutes.

(b) *Acetylcholine sensitivity.* Doses of novocain of the same order as those required to abolish fasciculation reduced the response of the muscle to acetylcholine but not proportionately to their effect on the response to nerve stimulation. Fig. 3 shows the isometric record of the gastrocnemius of an atropinized cat. At each of the arrows a dose of 50γ of acetylcholine was injected intra-arterially. Between the first two panels 2 mg. of novocain were injected. The second acetylcholine response is 3′ and the third 13′ after the administration of novocain.

(c) *Effect of novocain on tetanic responses.* Frequently in eserinized animals the tetanus produced by nerve stimulation at 60–100/sec. was not maintained beyond the first few impulses. This was attributable to the curare-like action of liberated acetylcholine. With uneserinized animals this effect was not obtained. After the administration of novocain to the latter animals the tetanus produced by stimulation of the motor nerve at 60–100/sec. was not maintained, relaxing after the initial twitch to a variable degree depending on the dosage. The twitch tetanus ratio was usually reduced, but the post-tetanic potentiation of the muscle twitch did not appear to be affected by the novocain. In this respect the results are in agreement with those of Harvey (1939).

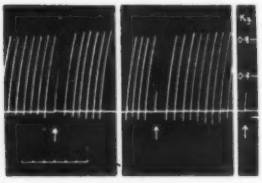

Fig. 3. Isometric record of innervated gastrocnemius muscle of a spinal cat. Maximal break stimuli were given at approximately 20" intervals and 50γ of acetylcholine at the arrows. Between the first two panels 2 mg. of novocain were injected intra-arterially. Time in half minutes. Other details in text.

(4) *The Action of Novocain on Denervated Muscle.*

(a) *Fibrillation.* The injection of saline intra-arterially and the occlusion of the circulation for 1 minute had no effect on the fibrillation of the denervated muscle but the injection of 2 to 10 mg. of novocain caused an immediate though usually transient cessation of fibrillation which returned in 3 to 5 minutes. For instance in one experiment 2 mg. of novocain reduced the rate and apparent "intensity" of fibrillation and 10 mg. abolished it completely for 3 minutes. After its return 20 mg. abolished it for a further 3 minutes. When it began to return, a further 50 mg. abolished it for a period of 45 minutes. Generally speaking the fibrillation was more resistant to the action of novocain than was physostigmine fasciculation, and the effect of doses of from 2–20 mg. was frequently more transient on the former than on the latter.

(b) *Effect on direct stimulation.* Doses of from 2–10 mg. had usually little or no effect on the magnitude of twitch due to direct stimulation of the muscle but sometimes such doses did reduce the magnitude of the response to direct stimulation. Larger doses reduced the twitch tension, but doses of the order which abolished indirect stimulation never reduced the response of the denervated muscle more than 50 p.c. In general it can be stated that novocain is not without direct action on the muscle itself, but such effects require larger doses than are required to produce similar effects in the innervated muscle. Harvey (1939) stated that novocain had no effect on the direct stimulation of denervated or curarized muscle although one of his figures does not appear to agree with that statement. My results are more in agreement with those of Macgregor who described a reduction of 10–20 p.c. in the twitch tension of denervated or curarized muscle with doses of 2–10 mg. of procain (novocain) or cocaine injected intra-arterially into the external iliac artery.

After doses of from 10–50 mg. the tetanus tension was not maintained and the twitch tetanus ratio was reduced. The post-tetanic enhancement of the twitch was unaffected. Fig. 4 shows the effect on the tetanic response of a denervated gastrocnemius. Between the panels, 10, 50 and 100 mg. of novocain were given intra-arterially.

(c) *Effect on the response of acetylcholine.* The action of novocain on the response of denervated muscle to acetylcholine was much less in evidence than its effect on the innervated muscle. In this respect it resembles curare. Fig. 5 shows 3 doses of 0·5γ of acetylcholine intra-arterially injected into a denervated gastrocnemius. Between the panels, 10 mg. and 20 mg. of novocain were given respectively. Responses to acetylcholine were reduced both in height and in the rate of development of tension.

These results confirm the observations of previous workers on the curare-like action of novocain. This was seen in its effects on the acetylcholine responses of denervated and innervated muscles, on the indirectly excited tetanus of innervated muscle and on its production of neuromuscular block although a direct action on the nerve possibly contributes to this last action. In addition a further curare-like action has been observed—the action on fasciculation. Apart from its curare-like action, the drug in larger doses has also a direct effect on the muscle itself. The results in this respect are more in agreement with those of Macgregor than Harvey. Thus novocain reduces the response to direct stimulation, reduces the twitch tetanus ratio, causes failure of maintenance of tetanus and abolishes fibrillation in denervated muscle.

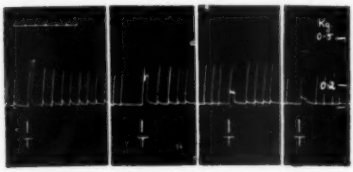

Fig. 4. Isometric record of gastrocnemius muscle denervated 12 days previously, maximal break stimuli being given at approximately 20″ intervals and tetanizing stimuli at T. Time in half minutes. Details in text.

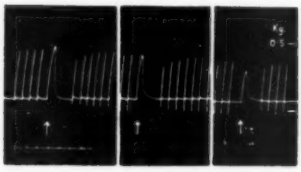

Fig. 5. Isometric record of a gastrocnemius muscle denervated 18 days previously, maximal break stimuli being given at approximately 20″ intervals, and 0·5γ acetylcholine at the arrows. Time in half minutes. Details in text.

DISCUSSION.

These results indicate the time relationship in denervated muscle of the loss of physostigmine fasciculation, the onset of fibrillation and the development of acetylcholine sensitivity. They confirm the observation that a functioning motor nerve is essential for physostigmine fasciculation because it is no longer obtained when the muscle becomes inexcitable by single stimuli applied to its nerve—in these experiments between 48 and 64 hours. Lissak, Dempsey and Rosenblueth (1939) have shown that the transmission of impulses from nerve to muscle may occur up to 77 hours after denervation if tetanizing stimuli are used. At all events the loss of fasciculation appears at an early stage of nerve degeneration whereas fibrillation and the high degree of acetylcholine sensitivity do not appear until about two days after neuromuscular block is complete for all forms of stimuli.

These observations are related to the problem of the mechanism of action of physostigmine in the production of fasciculation. The following views may be postulated. (1) Its action is one of potentiation enabling otherwise subminimal random impulses from the cut end of a divided nerve to produce fascicular twitching in the muscle. (2) It may be an anti-esterase action potentiating the effect of an otherwise subminimal amount of acetylcholine present in the nerve endings. (3) It may have a direct stimulant action on the nerve terminal itself.

So far as the first view is concerned I have shown in a few experiments that fasciculation is unaffected by a complete nerve block by cocain. In addition fasciculation was obtained 40 hours after nerve section. As Eccles (1936) points out referring to one of Langley and Kato's (1915) experiments in which fasciculation was obtained 29 hours after denervation, it is unlikely that random impulses would still pass down from the cut end after such intervals. Moreover, there is no reason why the effect of random stimuli should be abolished by curare or by novocain in doses too weak to block neuromuscular transmission.

Similar doses of curare or novocain are required to abolish fasciculation on the one hand and the response of innervated muscle to acetylcholine on the other. This would appear to favour the second view described above. Lissak, Dempsey and Rosenblueth (1939) have shown that following denervation neuromuscular transmission ceases before nerve conduction, and from their experimental results they consider that the former is due to a loss of acetylcholine from the

nervous tissue. If the second view is correct such a loss may explain the failure to obtain fascicular twitching in denervated muscle at an early stage of nerve degeneration. Nevertheless, in the absence of impulses from some source it is difficult to see how such acetylcholine present within the nerve tissue could be made available for stimulation under the potentiating action of eserine. Moreover, after 5 days of denervation the muscle is sensitized some hundred-fold to the action of acetylcholine—a degree which should more than make up for any possible reduction in the local acetylcholine content of the tissues.

Eccles (1936) reviewing the evidence concluded that eserine acted on the muscle itself, i.e. on the end plate region. Masland and Wigton (1940) showed that during prostigmine fasciculation antidromic impulses arise from the nerve terminations. It would appear from such an observation that prostigmine and allied drugs stimulate that portion of the end plate which is derived from the nerve axon. Nevertheless, the view that eserine stimulates the nerve endings is not devoid of difficulties. It is difficult to see why a small dose of novocain or curare should abolish fasciculation without blocking neuromuscular conduction unless these drugs act not only at the neuromuscular junction but directly antagonize the stimulating action of physotigmine on the nerve termination itself. That this may be the case is indicated by the fact that curare not only abolishes fasciculation in the muscle but also the accompanying antidromic impulses (Masland and Wigton, 1940). Masland and Wigton found that acetylcholine intra-arterially injected also caused antidromic impulses and they considered that it acted on the nerve terminations. If this is so it would provide an explanation for the fact that similar doses of curare or novocain can abolish both fasciculation and acetylcholine responses without affecting neuromuscular transmission. At all events an explanation of physostigmine fasciculation based only on anti-esterase activity will not suffice. Whatever may be the mechanism of action of physostigmine functioning motor nerve terminals are essential.

Attempts to extend this view by pharmacologically "removing" the nervous tissue of a muscle by means of novocain intra-arterially injected were unsuccessful because in confirmation of Harvey's (1939) work it was shown that novocain resembled curare in certain actions. In addition it was shown that like curare it abolished fasciculation in doses too small to effect neuromuscular transmission.

Unlike Harvey and more in agreement with Macgregor (1939) it was found that novocain in suitable dosage had an action on the directly excited twitch and tetanus of denervated muscle. As a corollary to this observation it was shown that novocain in suitable dosage abolished the fibrillation in denervated muscle. It should be pointed out that Harvey used a close intra-arterial injection and did not allow the drug to remain in contact with the muscle. Macgregor used distant intra-arterial injections from the iliac artery. In these experiments an "adjacent" technique was adopted and the novocain left in contact with the muscle for 30-60 seconds. These facts probably account for the slight differences in the results obtained and the discrepancies in the doses employed.

These results also show that the high acetylcholine sensitivity appears at the same time as the onset of fibrillation, i.e., 5 days after denervation. This cannot be taken to mean, however, that this spontaneous movement is produced by the action of acetylcholine on the sensitized muscle for in one and the same muscle curare reduces the response to acetylcholine without affecting the fibrillation. Moreover, it has been shown that eserine has little or no action on the fibrillation (Langley and Kato, 1915) (Rosenblueth and Luco, 1937). Possibly some surface change in the muscle is responsible for both the acetylcholine sensitivity and the continuous spontaneous activity.

SUMMARY.

The high degree of sensitization (some hundred-fold) of denervated muscle to acetylcholine develops after about 5 days of denervation. Contractural responses may be seen as early as the third day.

In confirmation of other workers fasciculation cannot be produced by physostigmine after about 2 days and spontaneous fibrillation does not appear till after 5 days of denervation.

Fibrillation is unaffected by doses of curare which in the same muscle reduce the response of denervated muscle to acetylcholine.

Novocain in addition to its known curare-like actions abolishes fasciculation in doses which have little or no effect on neuromuscular condition. It also has a direct action on denervated muscle and can cause cessation of fibrillation.

The nature of fasciculation is discussed.

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THE CHANGE IN THE ADENYL CONTENT OF SKELETAL MUSCLE AFTER DENERVATION

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The changes in the content and distribution of the various chemical constituents of skeletal muscle following denervation have been the subject of numerous papers, Hines and Knowlton (1933), Chor, Dolkart and Davenport (1937), Tower (1939).

There are 4 groups of substances: (1) General protoplasmic constituents whose percentage content in the muscle mass remains the same during the course of atrophy, e.g., total nitrogen, non-protein-nitrogen and water, (2) Specific muscle constituents which are lost at the same rate as the muscle substance itself, e.g., potassium and creatine, (3) Specific muscle constituents which are lost at a greater rate than the muscle substance, e.g., creatine-phosphate and glycogen, and (4) Substances related to the connective tissue whose percentage content in the muscle mass rises during atrophy, e.g., inorganic phosphate and calcium.

The adenyl content of denervated muscle does not seem to have been determined by direct estimation but has been obtained indirectly from phosphorus determinations. Such methods led Hines and Knowlton (1933) to the conclusion that the phosphate fraction attached to the adenine group disappeared from denervated muscle at a rate which closely paralleled the rate of loss of muscle substance. It seemed worth while to determine the change in the active adenyl content of skeletal muscle after denervation, using the method of Drury, Lutwak-Mann and Solandt (1938).

METHODS.

A sciatic nerve of each of 50 adult rats was aseptically divided high in the thigh and a piece 0·5 cm. long removed. In half of the animals the right and in the other half the left nerve was divided. The animals were killed from 3 days to 8 weeks after denervation, and the gastrocnemius muscles dissected out and weighed. The entire muscle or a weighed portion of it was ground up with powdered quartz in normal saline, muscle being present in the extracts so made in the proportion of 1: 20. In order to control the factor of enzyme activity during extraction the period of the latter was always five minutes, after which the extract was boiled and filtered. The activities of the filtered extracts of each muscle pair were compared with each other and with the activity of adenosine (B.D.H.) using as a test object the heart of the atropinized guinea-pig (Drury, Lutwak-Mann and Solandt, 1938).

RESULTS AND DISCUSSION.

There are several adenyl compounds which may be present in muscle extracts. Adenyl pyrophosphate breaks down into adenylic and phosphoric acids during muscular activity. A similar break down occurs during neutral or alkaline hydrolysis; acid hydrolysis yields adenine, ribose phosphate and phosphoric acid (Lutwak-Mann, 1939). The pharmacological activity of adenyl compounds depends on (1) the adenine ribose complex, adenine itself being inactive and (2) the integrity of the amino group of the purine. A deaminase in heart muscle (Ostern and Mann, 1933), in serum (Drury *et al.*, 1938), and in small amounts in skeletal muscle (Ostern and Mann, 1933) can inactivate adenyl compounds.

The method of estimation of active adenyl compounds depends on the depression which they produce in the rate and amplitude of the beat of the auricle of the atropinized guinea-pig. Since every adenyl compound does not exhibit the same degree of activity, a value for the total activity of an extract expressed in terms of adenosine does not necessarily represent the total quantity of adenyl compounds present, nor does a similar value for different extracts necessarily represent the same quantity of active adenyl compounds in each. Thus it is conceivably possible that a loss of total active adenyl compounds from a muscle could be masked by an increase in the proportion

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TABLE 1.

Duration of denervation in days.	Muscle weights $\frac{D}{N}$ p.c.	Adenosine per gm. $\frac{D}{N}$ p.c.	Total Muscle tissue $\frac{D}{N}$ p.c.	Total Adenosine $\frac{D}{N}$ p.c.
3	98·5	99·3	98·4	98·5
4 experiments	(97 — 100)	(97 — 100)	(96 — 100)	(97 — 100)
7	83·4	94·0	80·5	79·4
7 experiments	(76 — 97)	(87 — 100)	(72 — 90)	(72 — 88)
14	59·0	90·5	51·8	53·4
8 experiments	(52 — 63)	(67 — 100)	(47 — 58)	(35 — 64)
21	49·0	68·3	40·0	33·6
7 experiments	(45 — 67)	(62 — 76)	(32 — 61)	(28 —45)
28	39·0	72·0	28·3	26·8
5 experiments	(33 — 45)	(44 — 100)	(21 — 35)	(15 — 44)
35	37·7	60·3	26·7	22·3
3 experiments	(27 — 45)	(50 — 71)	(14 — 35)	(19 — 27)
39	29·0	64·0	16·5	19·0
1 experiment			(4·8)*	
49	23·8	49·4	10·4	11·8
5 experiments	(20 — 28)	(34 — 63)	(9 — 15)	(7 — 15)
			(5·8)*	
56	24·6	55·2	11·2	13·6
5 experiments	(20 — 28)	(50 — 63)	(6 — 15)	(10 — 18)

* See text, p. 199.

of more active adenyl compounds. For instance, in view of the continuous fibrillation, one might expect less adenyl pyrophosphate and more adenylic acid in the denervated than in the innervated side. It is probable however as Drury (1936) indicates that during the preparation of extract most of the pyrophosphate is in any case broken down to adenylic acid. Other alterations in the constitution of the adenyl compounds during extraction should obtain to the same degree for both innervated and denervated sides. If these facts are correct, it should be legitimate to consider the extracts of innervated and denervated muscle as strictly comparable and to regard activity as an estimate of the adenylic acid present.

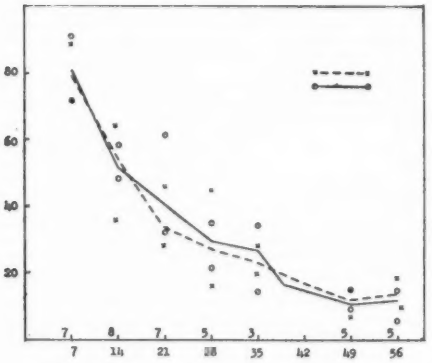

Fig. 1. Graph showing the percentage of original adenyl content (broken line) and percentage of original muscle tissue (continuous line) in the gastrocnemius muscle of the rat, 7 to 56 days after denervation. The curves pass through points which represent the mean values of each group. The other points represent the range and the figures the number of animals in each group. Ordinate in percentage, abscissa in days.

Because all of the adenyl pyrophosphate may not be broken down to adenylic acid during extraction, experiments were performed in which (1) muscle extracts were compared with one another and with adenosine and (2) a portion of each extract was autoclaved at 120° C. for 10–30 minutes and again similarly tested. The extracts were always neutral or slightly alkaline to litmus but autoclaving, which should break down any pyrophosphate present in such extracts to adenylic acid, had no influence on their activity.

The content of adenyl compounds in mg. per gm. of muscle (expressed as adenosine) of 40 innervated muscles ranged from 0·15 to 0·32 mg. with a mean of 0·24 mg. and a standard deviation of 0·047 mg. Following the practice of Bennet and Drury (1931) these figures should be multiplied by ½ to represent mg. of adenylic acid per gm. of tissue.

The results are set out in Table 1. The concentration of adenosine in each muscle decreased as the atrophy increased reaching approximately 50 p.c. of the original value after seven or eight weeks, atrophy

having occurred to about 24–25 p.c. of the original weight by that time (see columns 2 and 3). Stimulation of the divided nerve showed no evidence of nerve regeneration. The whole muscle, however, is not made up of muscle tissue, between 10 and 20 p.c. consisting of connective tissue and vessels. Hines and Knowlton (1933) chose an arbitrary figure of 15 p.c., i.e. the percentage of losable weight in an innervated muscle is 85 p.c. The figures in column 4 have been calculated on this basis. For example, if the denervated muscle had originally the same weight as its normal fellow and is now 50 p.c. of the original weight, the percentage of the original muscle substance remaining would be $\frac{(50 - 15)}{85}$ 100. Assuming that the content of adenyl compounds was originally the same on each side, and expressing the remaining adenyl compounds in the denervated muscle as a percentage of the original value, figures are obtained which closely parallel the percentage of the initial losable weight (or muscle substance) still remaining (see columns 4 and 5). The lower figures in each row in the table represent the range of values in each group. The upper figures in each row represent the mean for each group. The results are graphically set out in Fig. 1 and a typical titration is seen in Fig. 2.

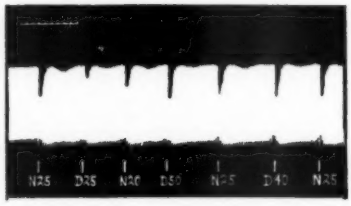

Fig. 2. Responses of the right auricle of an atropinized guinea-pig to extracts of rat muscle. At N extracts of innervated muscle and at D extracts of muscle 28 days after denervation were injected. The figures are the number of milligrams of muscle represented by each injection. Time in half minutes.

The value of 15 for the percentage of non-losable weight is arrived at from the observations of numerous observers on the ultimate degree of atrophy in rat muscle. Should 20 p.c. for example be more accurate for the animals used here, the results, within the limits of experimental error, would be substantially the same for the first 5 weeks after denervation, but with longer periods the percentage remaining of the original adenyl content would become greater than the percentage remaining of the original losable weight (see the figures marked with an asterisk in column 4 of the Table). On a 15 p.c. basis this discrepancy is only slight. In view of the fact that at 7 and 8 weeks the atrophy time curve has flattened out and in the absence of nerve regeneration atrophy has reached 75 p.c., 20 p.c. is possibly a more accurate value than 15 p.c. for the non-losable muscle weight in these animals. At all events, a departure from the parallelism of the earlier stages is readily accountable for by the large proportion of nuclei in the degenerated muscle. These should be a rich source of adenyl compounds.

SUMMARY.

The active adenyl compounds of denervated muscle are lost from the muscle at the same rate as is the muscle tissue itself. Thus they behave similarly to certain other muscle constituents such as creatine and potassium. In extreme atrophy there is possibly a greater percentage of adenyl content than of original muscle tissue. This could be accounted by for the relative increase in nuclei in the denervated muscle.

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THE SYNTHESIS OF POLYSACCHARIDES BY YEAST CELLS FERMENTING ANAEROBICALLY GLUCOSE, FRUCTOSE OR MANNOSE.

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In a previous paper (Gottschalk, 1941) a complete balance of the anaerobic fermentation of glucose by yeast cells comprising determinations of the carbon dioxide and alcohol produced and of the fate of the sugar consumed but not fermented has been described. The results of the experiments demonstrated that under these conditions only 77 p.c. of the glucose utilized is fermented to CO_2 and ethanol, the ratio alcohol : CO_2 being 1·03. Two-thirds of the glucose consumed but not fermented is reconvertible into reducing substance by acid hydrolysis. Rather more than half of the non-fermented glucose is built up to polysaccharides, mainly glycogen and gum, besides smaller amounts of membrane polyose. In this paper a comparative investigation of the relation between synthesis of polysaccharides and alcoholic fermentation with glucose, fructose and mannose as substrates has been carried out under anaerobic conditions.

METHODS.

The experiments were performed at 25° C. with fresh compressed baker's yeast (Effront or Hudson), suspended in M/15 KH_2PO_4 solution. The CO_2 produced under strictly anaerobic conditions was measured in the apparatus described recently (Gottschalk and Rawlinson, 1942). With glucose as substrate fermentation was allowed to go to completion; under the prevailing conditions this was attained in about 140 minutes. With fructose and mannose as substrates even after 3 hours small amounts of the sugar were left. These experiments were interrupted after 140, 150 or 180 minutes. At the end of the experiments the yeast suspension was siphoned into a cooled pyrex centrifuge tube (20 mm. × 110 mm.), frozen and centrifuged as described previously. The clear supernatant fluid was decanted and a determination of its reducing power made by the Hagedorn-Jensen micromethod. In the yeast cells the polysaccharides (glycogen, gum, membrane polyose; cf. McAnally and Smedley-MacLean, 1935 and 1937) were determined according to Pflüger's method adapted to the special characteristics of the yeast polysaccharides and using suggestions of Good, Kramer and Somogyi (1933): Into the pyrex tube containing the 0·4 gm. centrifuged yeast were put 1·0 c.c. distilled water and 1·5 c.c. 60 p.c. KOH (final concentration = 30 p.c. KOH). The tube with the well-mixed suspension, fitted with an air reflux condenser, was immersed in a boiling water bath for 3 hours. After cooling 1·0 c.c. distilled water and 5·0 c.c. absol. alcohol were added. This mixture was heated in the water bath till it started to boil, then cooled, centrifuged and the supernatant fluid decanted. The precipitate sticking to the glass wall was washed once with 60 p.c. alcohol on the centrifuge. After decanting, the last of the alcohol was removed by heating the tube for a minute in the water bath. Three drops of 2 N HCl and 4 c.c. N HCl were added to the precipitate and the pyrex tube, fitted with an air reflux condenser, heated for 2–2¼ hours in a boiling water bath. After cooling and neutralization with N NaOH (phenol red as indicator), the final adjustment to a faint red colour being made with 0·1 N NaOH, the fluid was made up to a suitable volume and analysed for its reducing power according to Hagedorn-Jensen. The polysaccharide content of the control was similarly determined.

The application of higher concentrations of KOH than 30 p.c. final concentration has been tested, but it did not increase the yield of polysaccharides.

In a series of experiments to test the method the reducing substances in the polysaccharide hydrolysate were determined before and after yeast fermentation. The figures in Table 1 demonstrate that the acid hydrolysis of the yeast polysaccharides yields only negligible quantities of non-fermentable matter oxidizable by potassium ferricyanide. Glucose puriss, added to the polysaccharide hydrolysate after removal of the fermentable substances by yeast, is quantitatively recovered. Thus, in this case the Hagedorn-Jensen method can be applied to the sugar determination (cf. also Elliott and Schröder, 1934).

TABLE 1.

Experiments on the determination of yeast polysaccharides (modified Pflüger's method).

The results are expressed in terms of glucose.

Amount of fresh yeast analysed. mg.	Total reduction. mg.	Non-fermentable reducing matter. mg.	Fermentable sugar. mg.
1,000	55	2	53
1,000	58	2·5	55·5
500	25	1	24
500	27	1	26
500	28	1	27
After fermentation of the polysaccharide hydrolysate 25·8 mg. glucose puriss. added.			
	27·2	1	26·2

The value of the "total carbohydrates" of fresh compressed yeast, obtained by hydrolyzing the living yeast directly with N HCl for 4 hours in a boiling water bath, is much higher than the polysaccharide content. 500 mg. fresh yeast (Effront) yielded an average of 53 mg. total carbohydrate (as glucose and corrected for the gradual loss previously shown to occur during hydrolysis with N HCl; Gottschalk, 1941) of which 46 mg. were fermentable and 7 mg. non-fermentable. The difference between the "total carbohydrate" (after allowing for the non-fermentable reducing matter in the hydrolysate) and the polysaccharides is due in part to the presence of trehalose in compressed yeast.

TABLE 2.

Balance of the anaerobic fermentation of glucose, fructose and mannose respectively by compressed yeast.

400 mg. fresh baker's yeast suspended in M/15 KH_2PO_4 solution; 147·3–153·1 mg. hexose added. Total volume: 15·0 c.c. pH of the suspension at the beginning of the experiment: 4·3. Temperature: 25° C. Gas-space: nitrogen (free from oxygen). Fermented to the complete or nearly complete consumption of the sugar added.

The amounts of polysaccharides are expressed in mg. glucose.

No.	Yeast.	Dry weight of the yeast added. mg.	Time in min.	Hexose added.		Hexose recovered. mg.	CO_2 produced. c.c.	Polysaccharides of the yeast cells. Control.	After fermentation.
1	E	107·8	140	glucose	149·2	0	28·5	18·8	33·9
2	E	107·8	140	fructose	152·0	4·2	28·5	18·8	34·0
3	E	114·5	140	fructose	149·1	4·8	27·6	19·2	35·0
4	H	112·0	140	glucose	149·8	0	28·6	24·8	42·2
5	H	112·0	140	fructose	153·1	4·8	28·4	24·8	43·5
6	H	110·0	140	glucose	150·8	0	28·7	26·0	44·5
7	H	110·0	140	fructose	152·7	5·2	28·3	26·0	43·7
8	E	116·0	140	glucose	147·3	0	27·6	20·0	35·5
9	E	116·0	180	mannose	151·4	1·4	28·0	20·0	34·9
10	E	113·8	140	glucose	151·2	0	28·5	20·2	37·2
11	E	113·8	150	mannose	152·1	2·1	28·6	20·2	35·4

The experimental results recorded in Tables 2 and 3 demonstrate that with glucose, fructose or mannose as substrate on an average 76·5 p.c. of the hexose consumed is fermented to CO_2 and alcohol (cf. Meyerhof, 1936; van Niel, 1941). 40–55 p.c. of the sugar consumed but not fermented is synthetized to polysaccharides. Whether glucose, fructose or mannose be the substrate there is no marked difference in the quantity of polysaccharides formed by a given strain of yeast. The ratio of this increase in polysaccharides to hexose consumed but not fermented varies, however, with different strains of yeast. Between 9·9–12·6 p.c. of the total amount of hexose consumed is built up to polysaccharides.

TABLE 3.

Summarizing the experimental results shown in Table 2.

No. of experiment shown in table 2.		Total hexose consumed.	Hexose fermented.	Polysaccharides formed during fermentation.	Hexose fermented / Total hexose consumed.	Polysacch. formed / Hexose consumed but not fermented.	Polysacch. formed / Total hexose consumed.
		mg.	mg.	mg.	p.c.	p.c.	p.c.
1	G	149·2	114·6	15·1	76·8	43·6	10·1
2	F	147·8	114·6	15·2	77·5	45·8	10·3
3	F	144·3	110·9	15·8	76·9	47·3	10·9
4	G	149·8	115·0	17·4	76·8	50·0	11·6
5	F	148·3	114·2	18·7	77·0	54·8	12·6
6	G	150·8	115·4	18·5	76·5	52·3	12·3
7	F	147·5	113·8	17·7	77·2	52·5	12·0
8	G	147·3	111·0	15·5	75·4	42·7	10·5
9	M	150·0	112·6	14·9	75·1	40·0	9·9
10	G	151·2	114·6	17·0	75·8	46·4	11·2
11	M	150·0	115·0	15·2	76·7	43·4	10·1

SUMMARY.

Compressed yeast, suspended in phosphate solution (pH = 4·3), with glucose, fructose or mannose as substrate ferments anaerobically to CO_2 and alcohol only 76·5 p.c. of the sugar consumed. About half of the hexose utilized but not fermented is synthetized to polysaccharides. The ratio

$$\frac{\text{polysaccharides formed}}{\text{hexose consumed but not fermented}}$$

varies somewhat with different strains of yeast; but there are no marked differences in this ratio with different hexoses.

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THE IODINE NUMBERS OF THE LIVER LIPOIDS IN EXPERIMENTAL LIVER DAMAGE

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In a previous publication, Ennor (1942), it was shown that the livers of guinea pigs treated with phosphorus and carbon tetrachloride showed no impairment in their abilities to oxidize either the fat originally present or added fatty acids. Winter (1938) states that the mechanisms concerned with desaturation of fatty acids in the livers of dogs poisoned with carbon tetrachloride are interrupted and that this is responsible for the decrease in the unsaturated fatty acid content of the plasma. Such a finding, of course, would mean that animals so treated would have an impaired ability to oxidize fat and is suggestive, in view of my earlier results, of a species difference in the response towards such toxic agents. This problem forms the basis of the present investigation.

EXPERIMENTAL.

Male guinea-pigs were used throughout. The same system of experimental numbering has been preserved and the details of diet, treatment, etc., will be found in my earlier communication.

The iodine numbers of the fats were determined by the method of Dam as described by Plimmer (1938) upon the fatty residue obtained after evaporation of the alcohol-ether solution to dryness in a current of carbon dioxide. "Fat" refers to the total material extractable from the livers by means of a 70–30 alcohol-ether mixture.

The results obtained in a series of normal and treated animals are set out in Table 1.

TABLE 1.

Normal animals.			Animals treated with carbon tetrachloride.		
Exp. No.	Fat gm. p.c.	I_2 No.	Exp. No.	Fat gm. p.c.	I_2 No.
61	5·5	57	21	11·5	88
62	5·9	64	18	12·4	87
63	5·3	58	25	15·0	96
64	6·1	60	26	13·0	94
65	5·4	52	27	8·4	75
66	5·7	66	28	7·6	83
67	6·1	73	29	6·0	71
68	5·5	53	30	11·4	98
69	6·3	59	31	7·3	78
70	6·7	58	32	9·6	88
38	8·2	67	33	11·9	84
40	6·6	70	34	10·5	81
45	6·4	70	42	10·9	75
46	6·5	78	43	10·7	90
47	5·3	54	44	19·2	94
		—	48	12·3	63
	Mean	63	49	8·9	77
			51	11·6	82
					—
				Mean	84

It is apparent from the above figures that the iodine number of the liver fat from the poisoned animals is actually greater than that from the normal controls. This result was obtained by determination of the iodine uptake of fat whilst Winter carried out all his investigations upon fatty acids. In order to obtain a strict comparison between this worker's results and those quoted

[1] Working under a full time grant from the National Health and Medical Research Council, Australia.

here it was decided to carry out a series of investigations on the fatty acids separated from the fat residue after evaporation of the alcohol-ether solvent.

The procedure adopted was as follows: A weighed amount of finely ground liver was extracted with several successive small volumes of a warmed 70–30 alcohol-ether mixture and the extract filtered. The solution was maintained at 50–60° C, a stream of carbon dioxide or nitrogen passed through to remove the ether, and the resultant solution made 2 N with respect to NaOH. The alkaline alcoholic fatty solution was then boiled under a reflux condenser for 1½–2 hours. Following saponification the non-saponifiable material was extracted with successive amounts of petroleum-ether (B.P. 30–50° C.). The resultant solution, after acidification, was again extracted with petroleum-ether and the fatty acids so removed. This fatty acid solution was allowed to stand over anhydrous sodium sulphate for 24 hours and then filtered into a tared flask. After removal of the petroleum-ether by distillation the fatty acid residue was weighed, redissolved in carbon tetrachloride, made up to known volume and aliquots withdrawn for determination of the iodine uptake.

All animals used were well-fed male guinea-pigs and those which were treated with carbon tetrachloride received subcutaneously 0·15 ml. of the chlorinated hydrocarbon, diluted with an equal volume of olive oil, on each of 3 successive days and were killed on the fourth day. Such treatment has already been shown to result in marked hepatic damage.

The results of this series are shown in Table 2.

TABLE 2.

Normal animals.		Animals treated with carbon tetrachloride.	
Exp. No.	I_2 No.	Exp. No.	I_2 No.
71	91	72	103
81	85	73	99
82	100	75	101
83	103	76	114
85	97	77	124
	—	78	108
Mean	95	79	110
		Mean	108

The results shown in this Table lend additional support to the view already expressed that the iodine numbers of the liver fat are greater in poisoned animals than in the untreated controls.

DISCUSSION.

The initial steps in the oxidation of fatty acids, i.e., the transformation of the long chain acids to acetoacetic and β-hydroxybutyric acid, take place only in the liver and, following formation, they are transferred to extra-hepatic tissues for complete oxidation to carbon dioxide and water. If then, as a result of starvation or poisoning, fat accumulates in the liver in pathological amounts, it would be expected that the available enzyme centres would be saturated with substrate and the fat oxidized. The extent to which such an oxidation occurs would be reflected in the rate of ketogenesis. It has been shown (Ennor, 1942) by means of the tissue slice technique that the rate of production of ketone bodies is markedly greater in the carbon tetrachloride fatty liver than in the normal liver. In view of this, it would not be expected that the iodine numbers of the hepatic lipoids would be lower than normal although it is possible that lower values would be found in the very early stages of poisoning as a result of dilution of the liver fat with the less saturated fat transported from the body depots.

Whilst few details are available, the results of Leick and Winkler (1902) are of interest. These workers found that the iodine numbers of subcutaneous and myocardial fat of the normal dog were 56·1 and 70·7 respectively, whilst the fat from similar sources in the phosphorus-poisoned dog had iodine numbers of 58·6 and 82·9. In the case of a normal sheep the iodine numbers of the fat from the subcutaneous and myocardial regions were 38·2 and 58·2 respectively and the respective values for the fat from similar sources in a phosphorus-poisoned animal were 36·9 and 64·1. These values, whilst being too few in number to allow of definite conclusions, at least give no indication of any fall in the degree of unsaturation of fat from these sources in phosphorus poisoning.

Winter (1938) has found that the iodine number of the total plasma fatty acids in dogs are definitely lower after 10 weeks of carbon tetrachloride treatment. Investigating the effect of

treatment with the latter compound on rats, the same author (1939) comes to the conclusion that the hepatic fatty acids have a lower iodine number in the case of treated animals than in normal ones. The experimental results, however, are rather few in number, and the variation and overlapping of values seem too great to justify this statement. In the case of the carcass fatty acids, however, there is a very real difference—the treated animals having the lower iodine numbers. In the same communication the results of the investigations into faecal fatty acids are given. Here again the iodine number of these fatty acids are lower in the treated than in the normal animals. The fact that the ingestion of a diet containing greater amounts of unsaturated acids leads to a recovery to normal values in all these cases, does not seem of significance. In fact, if this result was not obtained it would be rather surprising since the indication would, of course, be that carbon tetrachloride treatment interferes with absorption of fat—a mechanism which has not been questioned.

Some iodine number determinations were carried out on the liver fat of male albino rats which received similar amounts of carbon tetrachloride as did the guinea-pigs. The results are shown in Table 3.

TABLE 3.

Normal animals.			Animals treated with carbon tetrachloride.		
Exp. No.	Fat gm. p.c.	I_2 No.	Exp. No.	Fat gm. p.c.	I_2 No.
4	4·9	81	1	11·7	86
5	4·9	87	2	10·9	87
6	5·0	93	3	18·2	84
7	5·6	66	10	13·1	80
9	5·5	75	11	11·6	83
		—	12	9·3	81
	Mean	80			—
				Mean	83

Consideration of the above results reveals no significant difference in the iodine number of liver fat from treated and normal animals. This finding is in agreement with that of Winter (1939), although this worker, as already mentioned, considers that the treated animals have a lessened hepatic content of unsaturated fatty acids—a statement which does not gain support from his experimental results.

There is, therefore, a very marked variation in the behaviour of the rat and guinea pig in so far as the degree of unsaturation of hepatic fat is concerned. Similar marked differences in the rates of oxygen consumption and ketone body production are well known to exist between the livers of these two species and these may possibly provide a clue to the differences in iodine numbers following carbon tetrachloride poisoning.

In view of the results recorded in the previous publication, the increased iodine number of the hepatic lipoids from guinea pigs treated with carbon tetrachloride can be explained by the increased rate of oxidation of the fat.

SUMMARY.

The iodine numbers of the total fat and fatty acids of the guinea-pig liver are increased following administration of carbon tetrachloride.

No such change is encountered in the case of the rat where the iodine number remains within normal limits. There is a species difference in the behaviour of rats, dogs and guinea-pigs following administration of carbon tetrachloride.

These findings are interpreted in the light of previous investigations.

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SEROLOGICAL PROTECTION AGAINST ARSENIC COMPOUNDS

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In general, it is relatively easy to sensitize experimental animals or human beings against simple chemical substances (cf. Landsteiner and Chase, 1941). Immunological protection against these substances has never been demonstrated, although serological reactions can be obtained *in vitro*. This latter phenomenon is well illustrated in the experiments of Landsteiner *et al.* on atoxyl-substituted proteins and in the recently published experiments of Hooker and Boyd (1940) on immunization against strychnine.

Butler, Harington and Yuill (1940) obtained protection against the temperature-lowering action of aspirin by a specific anti-aspiryl serum. Antigenic substances of low molecular weight are known to dissociate readily from the antigen antibody complex, and thus protection against a substance having such a low molecular weight as aspirin would be very remarkable. True serological protection occurs, as a rule, only when colloidal particles of larger dimensions are used as antigens. Further, true serological protection against a substance which contains only one determinant group is difficult to understand. All the available facts support the view that immunological reactions, apart from inhibition of serological reactions, can only occur if antigen and antibody have more than one reactive group. The object of the present work was to investigate the question of true serological protection with a group of compounds whose chemical and serological properties were well known and where all possibilities could be investigated with comparative ease.

The points to be determined were, (a) whether by increasing the molecular size it would be possible to obtain true serological protection against toxic substances having only one determinant group, and (b) whether true serological protection could be achieved when more than one determinant group was contained in the molecule. The substances chosen were *p*-amino-phenyl-arsenic acid and its derivatives.

METHODS AND MATERIALS.

Immune serum. Rabbits were immunized by bi-weekly intravenous injections of 2 ml. of an arsenic-ox-globulin preparation containing 3·5 p.c. protein and 0·07 p.c. arsenic. The serum was collected between the 6th and 9th weeks of the treatment, and merthiolate (Eli Lilly) was added as a preservative in a final concentration of 1/20,000. This serum gave a strong precipitin reaction with the homologous antigen in a dilution 1/25,000 (ring tests). Tested with an arsenic-rabbit-globulin preparation of the same composition the precipitin titre was 1/16,000. Absorption with optimal proportions of natural ox-globulin reduced the titre to 1/18,00 and 1/15,000 respectively. Only serum free from antibodies against unaltered ox-globulin was used in the protection experiments.

Arsenic Compounds.

1. Soamin (B.D.H.) para-amino-phenyl-arsenic acid.

2. Phenol-*p*-azo-phenyl-arsenic acid.

Para-amino-phenyl arsenic acid was dissolved in excess HCl, the solution cooled below 5° C. and diazotized by slow addition of $NaNO_2$. The resultant solution was slowly added to an alkaline solution of phenol, the mixture being stirred constantly and the temperature kept below 10° C.

The azo-compound was precipitated with 3 p.c. acetic acid, washed and dried at 100° C. Identical methods were employed for the preparation of the corresponding α and β-naphthol derivatives.

3. Resorcinol-tris(?)-azo-phenyl-*p*-arsenic acid. The preparation was probably a mixture of di and trisazo compounds. The method employed by Orndorf and Ray (1910) for the preparation of resorcin-trisazo-benzene was used. The compound was precipitated in a similar manner to the phenol derivatives, and dried at 80°.

Arsenic contents: Phenol derivatives, 20·1 p.c.; α Naphthol derivatives, 19·1 p.c.; β Naphthol derivatives, 18·2 p.c.; Trisazo derivatives, 26·1 p.c.

The toxicity of these compounds was determined on mice, the substances being dissolved in 0·5 ml. and injected intraperitoneally. The dose, in grams, which killed half of the mice within 4 days was as follows:

1. Soamin 0·005
2. Phenol 0·0025
3. α Naphthol 0·0025
4. β Naphthol 0·0025
5. Trisazo 0·015
6. Arsenic-ox-globulin solution 4 ml. (protein 7 p.c.; arsenic 0·14 p.c.)

The toxicity of the compounds 2–5 is slightly higher than that of soamin, probably because of an additive toxic effect of the phenol or naphthol moiety and that of the arsenic compound. The toxicity of the trisazo compound is only a third of that of soamin, and a further decrease in toxicity is observed in the case of the arsenic protein. Calculated on arsenic content the toxicity of the arsenic in the form of the different compounds would be for the compounds 2–4 in the order of 0·0005 gm., for the trisazo compound in the order of 0·0039 gm., and for the arsenic-protein in the order of 0·0056 gm.

PROTECTION EXPERIMENTS.

The protection experiments were arranged in such a fashion that one or multiples of one M.L.D. were injected intraperitoneally in batches of 4 mice, followed by subcutaneous injections of different amounts of serum. Controls received the same amount of normal rabbit serum. The

TABLE 1.

Trisazo compound:

1 M.L.D. = 0·015 gm. per 20 gm. mouse.

Immune serum 0·25 ml.	Controls.
1 7	1 2
2 S	2 4
3 S	3 4
4 S	4 S

1 M.L.D. = 0·015 gm. per 20 gm. mouse.

Immune serum 0·1 ml.	Controls.
1 S	1 3
2 S	2 3
3 S	3 3
4 S	4 S

1 M.L.D. = 0·015 gm. per 20 gm. mouse.

Immune serum 0·5 ml.	Controls.
1 4	1 3
2 S	2 4
3 S	3 6
4 S	4 S

2 M.L.D. = 0·03 gm. per 20 gm. mouse.

Immune serum 0·5 ml.	Controls.
1 2	1 3
2 5	2 3
3 5	3 3
4 7	4 3

2 M.L.D. = 0·03 gm. per 20 gm. mouse.

Immune serum 0·25 ml.	Controls.
1 1	1 2
2 5	2 2
3 5	3 3
4 5	4 4

Arsenic-ox-globulin:

1 M.L.D. = 4 ml. per 20 gm. mouse, given in 2 equal portions at 2 hours interval.

Immune serum 0·25 ml.	Controls.
1 7	1 5
2 S	2 6
3 S	3 6
4 S	4 7

Immune serum 0·5 ml.	Controls.
1 6	1 3
2 S	2 3
3 S	3 4
4 S	4 4

Immune serum 0·25 ml.	Controls.
1 S	1 2
2 S	2 3
3 S	3 4
4 S	4 S

Arsenic-rabbit globulin:

1 M.L.D. = 4 ml. per 20 gm. mouse injected as above.

Immune serum 0·25 ml.	Controls.
1 5	5 2
2 S	6 2
3 S	7 5
4 S	8 5

Immune serum 0·25 ml.	Controls.
1 3	5 2
2 6	6 3
3 S	7 3
4 S	8 3

animals were under observation for one week. The experiments with soamin, the phenol or naphthol derivatives will not be reported here because no protection could be obtained against these compounds.

Definite protection could be observed, however, against the trisazo compound and the arsenic-globulin.

In Table 1 the figures show the number of days between injection and death of the mice. The sign "S" indicates survival for more than 7 days.

IMPORTANCE OF DIFFERENT ANTIBODIES IN PROTECTION AGAINST ARSENIC-GLOBULIN.

Antisera against arsenic-globulin contain at least 3 different types of antibodies: 1. Antibodies directed against the determinant group proper. 2. Antibodies against chemically altered protein. 3. Antibodies against the unaltered protein.

In further experiments the importance of these 3 different types of antibodies for the protection obtained in the experiments with crude serum was investigated. For this purpose the serum was selectively absorbed with arsenic-rabbit-globulin in order to remove the antibodies against the determinant group only. The resultant exhausted serum still precipitated arsenic-ox-globulin.

TABLE 2.

Anti-arsenic-ox-globulin serum exhausted with ox serum and rabbit-arsenic-globulin, tested against 1 M.L.D. (4 ml.) arsenic-ox-globulin.

Immune serum 0·5 ml.	Controls.	Immune serum 0·5 ml.	Controls.
1 4	1 3	1 2	1 3
2 7	2 3	2 6	2 3
3 S	3 6	3 S	3 4
4 S	4 7	4 S	4 6

It appears, from the experiments reported in Table 2, that at least part of the protective action of the serum is due to the antibodies directed against the chemically altered protein. The amount of serum needed for protection is distinctly higher with the absorbed than with the unabsorbed serum. Exact evaluation of the part played by the different antibodies would require very large numbers of mice and was not thought to be sufficiently interesting to justify further elaboration.

SUMMARY AND CONCLUSION.

The factors determining serological protection were studied in experimental investigations carried out with arsenic compounds of different molecular weight and with a different number of arsenic groups per molecule.

As far as could be ascertained, molecular size has no influence on this effect, as no protection could be observed against arsenic compounds of larger type. Introduction of several determinant groups in one molecule, on the other hand, seems to have a decisive influence. Strongly marked serological protection could be obtained against resorcinol-trisazo-phenyl-*p*-arsenic acid and against arsenic-globulins.

The observations of Butler, Harington and Yuill (1940) prove that serological protection against certain pharmacologically active substances such as aspirin is obtainable even if the substance in question contains only one determinant group.

This apparent difference of results can be possibly explained by the fact that aspirin was used by Butler *et al*, and an organic arsenic compound by myself. In the case of our arsenic compounds, toxic substances must always result from cleavage in the body, whilst such a phenomenon would not occur when aspirin was used as the antigen. After these experiments were conducted, Hooker and Boyd (1941) published results of *in vitro* experiments with similar compounds. As no reaction could be obtained they interpret their results as a contradiction to the lattice hypothesis.

The protection experiments, on the other hand, show that true serological protection can be obtained against compounds which contain two or three determinant groups, which might not give *in vitro* reactions with antiserum. This seems im-

portant in connection with the question of antihormones where similar possibilities were lately discussed.

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STUDIES IN THE EPIDEMIOLOGY OF Q FEVER

10. THE TRANSMISSION OF Q FEVER BY THE TICK *IXODES HOLOCYCLUS* (WITH NOTES ON TICK-PARALYSIS IN BANDICOOTS)

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Ixodes holocyclus Neumann, referred to in Queensland as the scrub-tick, is the tick most commonly found attacking man in Australia. The distribution of this tick is restricted to a narrow coastal belt of country extending from northern Queensland to the New South Wales-Victoria border. Its most important native hosts appear to be bandicoots; *Isoodon torosus* in Queensland and *Perameles nasuta* in New South Wales.

In the known Q fever area, all of which (except Moreton Island) falls within the tick's range, bandicoots are proved reservoir-animals of infection (Derrick *et al.*, 1939; Derrick and Smith, 1940; Freeman *et al.*, 1940). Naturally infected scrub-ticks have not been found, but only the small number reported in this paper have as yet been examined; in the laboratory the tick has been found to act as a vector of infection.

Examination of Ticks from Bandicoots for Rickettsia burneti.

During a period of eleven months from October, 1937 to August, 1938, 58 bandicoots trapped in the coastal districts north of Brisbane were examined in the laboratory. Twenty-eight of these animals were carrying scrub-ticks. A total of 109 nymphal and adult ticks were inoculated into 23 guinea-pigs with negative results. In addition 111 larval ticks were inoculated; 65 ticks into 6 guinea-pigs and the remainder into 6 animals which also received nymphs and adults. No larvae were infected.

About seventy nymphs were removed while engorging from a bandicoot in July, 1938; from the host a strain of *R. burneti* was isolated (Derrick and Smith, 1940). Twenty-five of the nymphs were inoculated into a guinea-pig but failed to infect it. As the bandicoot's organs produced an *inapparent* infection in the primary guinea-pig and its serum contained *R. burneti* agglutinins it appears that these uninfected ticks attacked it after the phase in which its blood was infective. The vector responsible for the bandicoot's infection had departed prior to examination.

Technique.

A number of ticks, together with information relating to the methods adopted in raising them at the Commonwealth Serum Laboratories, Melbourne, and some canine anti-tick serum, were obtained through the courtesy of Dr. F. G. Morgan, Director of the Laboratories.

Bandicoots (*I. torosus*), upon which the ticks were freely placed, were the principal host animals used during most of the work. They were housed in cages over shallow trays of water into which the engorged ticks dropped and from which they were recovered by straining the water through muslin. Larvae but not nymphs fed readily upon guinea-pigs; a number of adults engorged on guinea-pigs when enclosed in capsules. The replete ticks were stored at room-temperature upon wet sand. In an endeavour to avert symptoms of tick-paralysis some of the bandicoots were inoculated with canine anti-tick serum.

Infection of Larvae and Transfer of the Virus to Nymphs.

Larval ticks were fed upon infected animals—guinea-pigs and a bandicoot—and examined for infection after ecdysis; a number of nymphs were found to be infected.

Three guinea-pigs were inoculated each with an infective dose of Q virus. A number of larvae were placed on each animal during the fourth day after inoculation. The guinea-pigs reacted with fever from the fourth to ninth days inclusive during which period the larvae fed, to fall replete from their hosts on the eighth, ninth and tenth days. The guinea-pig infections were later confirmed by the immunity of the animals to a second inoculation of virus.

The fed ticks were not examined prior to ecdysis but approximately thirty-nine days afterwards three of five unfed nymphs examined by smears contained abundant rickettsiae. A guinea-pig into which they were inoculated became infected with Q fever.

Bandicoot 253, a young male weighing 1,175 gm. was inoculated with 10 ml. of liver-spleen emulsion from an infected guinea-pig. The animal's normal temperature (< 98° F.) was exceeded on the third and fourth days after inoculation when the temperature rose to 98° and 99·4° F. respectively. *R. burneti* agglutinins, absent prior to inoculation, were observed in the animal's serum on the twenty-first day, and a rise in titre was noted when a further sample taken on the twenty-eighth day was examined. A number of larvae were placed on the animal during the second day after inoculation. They fell from the host on the seventh, eighth and ninth days. Although attached during the febrile period their engorgement was not completed until afterwards; many may not have taken up infective blood.

Approximately thirty-six days after ecdysis five nymphs were examined by means of smears; no rickettsiae were seen. A guinea-pig into which the ticks were inoculated did not become infected. As the ticks were then mixed with those which had become infected upon the guinea-pigs, proof that any had acquired infection was not obtained.

Twenty-five nymphs were fed upon each of two animals, Bandicoot 257 and Bandicoot 270; neither became infected. The majority of these nymphs, however, had fed as larvae upon Bandicoot 253 and as stated above may not have been infected. This is supported by the fact that none of twenty adults which developed from 41 recovered nymphs was found to be infected.

Infection of Nymphs and Transfer of the Virus to Adults.

Nymphal ticks were fed upon an infected bandicoot. When examined after ecdysis nine of twenty-nine female ticks were proved to be infected; none of nineteen male ticks was infected. Some of these adult ticks were fed upon two bandicoots one of which became infected with Q fever.

Bandicoot 252, a young male weighing 1,000 gm. was inoculated with 10 ml. of liver-spleen emulsion from an infected guinea-pig. About 150 nymphs were placed on the animal during the second day after inoculation. On the fifth, sixth and seventh days its temperature rose above normal. During this period the nymphs engorged, falling replete from the animal on the seventh, eighth and ninth days. The bandicoot's infection was confirmed by the detection of *R. burneti* agglutinins in serum taken on the fourteenth day, and the recovery of the virus on the fifteenth day from liver-spleen-kidney emulsion by means of guinea-pig inoculation.

One hundred and forty-two fed nymphs were recovered. Forty-one died without further development. Two guinea-pigs into which they were inoculated twenty-five days after engorgement died of a bacterial infection. The remainder of the ticks were not examined prior to ecdysis. Forty-eight were examined as adults by smears, smears and guinea-pig inoculation, or serial sections (Table 1). Nine of twenty-nine female ticks were found to be infected. Infection was not demonstrated in any of the nineteen male ticks examined.

A number of the female ticks were placed upon Bandicoot 255, a female of 1,395 gm., about three weeks after ecdysis. During the first day they had to be replaced repeatedly as they wandered or fell from the animal. On the second day six were seen to be attached; one (Z16) close behind the left ear and five (Z8, Z25, Z26, Z27 and Z34) across the shoulders. Five ticks dropped replete after periods of attachment ranging from seven to twelve days. Z8 was found dead and removed partly replete on the seventh day. Tick Z16 engorged most rapidly; it dropped on the eighth day, i.e. after seven days of attachment. Ticks Z25 and Z26 dropped replete on the ninth day, Z27 on the tenth day and Z34 on the thirteenth day.

On the ninth, tenth and eleventh days the animal exhibited severe symptoms of "tick paralysis"—paresis of the fore and right hind limbs together with respiratory embarrassment. The animal showed a marked improvement in condition during the succeeding two days and was normal on the next, the fourteenth day.

The bandicoot became infected with Q fever. The virus was recovered from its blood on the thirteenth day by means of guinea-pig inoculation. Blood drawn on the twentieth day was not infective. The infection of the animal was confirmed by the observance of a rise and fall in the titre of *R. burneti* agglutinins in samples of serum drawn at intervals from the tail. Clinically the infection was inapparent; the rectal temperature which was observed for a period of five weeks remained normal throughout.

All six ticks were examined by means of sections. Only one (Z16) was observed to be infected.

A number of the female ticks were placed upon Bandicoot 256, a male of 1,270 gm. On the second day eight ticks attached and began to engorge; one (Z17) above the right eye, two (Z19 and Z20) above the left eye, one (Z18) beneath the right ear and four (Z21, Z22, Z23 and Z24) beneath the left ear.

During the afternoon of the seventh day when the ticks Z17, Z18 and Z19 were almost replete the animal developed paresis in all four limbs. These ticks completed their engorgement during the night. The animal's condition became progressively worse on the eighth day and it died in the afternoon from respiratory paralysis. Two engorged ticks Z23 and Z24 and three partially fed ticks Z20, Z21 and Z22 were still attached at death. An emulsion of its liver, kidney and spleen was inoculated into two guinea-pigs; neither became infected with Q fever.

Of the eight ticks which fed upon Bandicoot 256 only one (Z21) was later proved to be

infected. However, the occurrence of infection in the others was satisfactorily excluded, by means of guinea-pig inoculation, only in the case of Z19; ticks Z18 and Z22 produced fatal bacterial infections in the guinea-pigs into which they were inoculated, and the remainder were examined by means of sections alone.

TABLE 1.

Examination of adult ticks fed as nymphs on an infected bandicoot.

Mode of examination.	Number of ticks examined.	Number of ticks with visible rickettsiae.	Number of guinea-pigs infected.	Number of ticks infected.
Smears	4 (2♂, 2♀)	–	–	–
Smears and guinea-pig inoculation	31 (17♂, 14♀)	7♀	7	7♀
Serial sections	13♀	2♀	–	2♀
Totals	48 (19♂, 29♀)	9♀	7	9♀

Infection of Adult Ticks.

Two adult ticks which fed upon an infected bandicoot were found to contain rickettsiae when tested thirty-four days after engorgement.

Two female ticks (Z75 and Z76) were placed upon Bandicoot 292, a male of 878 gm., which was inoculated two days later with 10 ml. of guinea-pig passage virus.

On the eighth day the animal became paretic in the hind limbs. Five ml. of canine anti-tick serum were inoculated. On the tenth day it was able to hop about with difficulty. At no time were respiratory symptoms apparent.

Blood taken on the ninth day, i.e. six days after inoculation, produced infection in a guinea-pig into which it was inoculated. On the following day the ticks fell from the bandicoot. Thirty-four days later they were each inoculated into a guinea-pig which became infected with Q fever.

Failure of Transovarial Passage.

Several hundred larvae, progeny of infected tick Z16, were fed upon each of two guinea-pigs. Neither of these nor another into which about seventy ticks were inoculated became infected. A similar number of larvae from infected ticks Z75 and Z76 were also inoculated into separate guinea-pigs with negative results.

The progeny of four ticks Z17, Z18, Z23 and Z26 not proved to be infected, but in which infection was not definitely excluded, were examined in a similar fashion. No evidence of infection in these ticks was obtained.

Distribution of Rickettsiae within the Tick.

Rickettsiae were observed in two of thirteen female ticks examined by means of serial sections. They were seen only in the lumen of the gut and its lining epithelium where they had invaded the cytoplasm but not the nuclei of the cells.

"Tick Paralysis" in Bandicoots.

I. holocyclus is responsible in Australia for the condition of "tick paralysis" in man and domestic animals. During this work it was found that bandicoots trapped in areas close to Brisbane, where scrub-ticks are rare if present at all, were susceptible, and larvae and nymphs as well as adults produced the disease when present in sufficient numbers.

Canine anti-tick serum was administered to three bandicoots in the hope of preventing "tick paralysis" from interfering with the Q fever experiments. None of the three died of "tick paralysis" although all became paresed. No conclusions regarding the protective value of the serum may be drawn in view of the small number of animals involved and the absence of adequate controls.

The cases of Bandicoots 255, 256 and 292 have been described. The following notes relate to two additional animals.

Two female ticks were placed upon Bandicoot 288, a young male of 570 gm., approximately three weeks after ecdysis. One attached at the base of the left ear, the other upon the outside of the right ear. The former tick was removed, dead, on the fourth day, showing no outward signs of having fed. On the same day five ml. of canine anti-tick serum were inoculated intramuscularly.

On the tenth day the animal became paretic in the hind limbs but continued to move about with some difficulty during this and the ensuing two days during which the tick completed its engorgement. At no time did it exhibit any respiratory symptoms. It appeared quite normal on the third day after the tick had fed. Six female ticks were placed upon Bandicoot 290, a female of 1,036 gm., approximately three weeks after ecdysis. The ticks attached immediately; one inside and two above the right ear, one above the left eye and two above the left ear.

On the fourth day ten ml. of canine anti-tick serum were injected subcutaneously. On the seventh day the animal exhibited signs of weakness and on the morning of the eighth day it was paretic in all four limbs and breathing slowly and irregularly with laboured gasps. Ten ml. of anti-tick serum were injected immediately. The animal's condition rapidly improved and at mid-day it was breathing quietly and regularly. An additional five ml. of serum were injected at 5 p.m. The ticks on this the eighth day were nearly replete and five of them dropped from the animal during the night. The remaining tick which appeared fully replete was removed the following morning.

On the tenth day it seemed probable that the animal would recover; respiration was normal; it was able to crawl, although slowly and with difficulty, and it showed some interest in its food. Unfortunately it was killed as a result of an accident. At autopsy apart from some congestion of the lungs the animal appeared normal.

All of five animals upon which adult ticks fed became affected. Bandicoots 288 and 292, upon which engorged one and two ticks respectively, became paresed only in the hind quarters; they showed no respiratory symptoms; both recovered. Six ticks fed on each of two animals; one, Bandicoot 252, recovered; the other, Bandicoot 290, died as a result of an accident just when its ultimate recovery seemed probable. Both of these animals and Bandicoot 256 which died following the feeding of eight ticks suffered respiratory paralysis in addition to paresis of both fore and hind limbs.

Upon Bandicoots 252, 257 and 270, were fed approximately 150, 25, 17 and later 8 nymphs respectively None of these animals were affected. Upon Bandicoot 294 were fed 528 nymphs. On the afternoon of the fourth day when many of the nymphs were nearly replete the animal was slightly paretic in the hind quarters. It was found dead on the following morning. Upon Bandicoot 293, fourteen days after the disappearance of symptoms following the feeding of larval ticks, were fed 485 nymphs. The animal displayed slight weakness in the hind quarters from the fourth to seventh days but was otherwise normal. Perhaps its survival, which contrasts strongly with the behaviour of Bandicoot 294, was due to some degree of immunity acquired through the previous feeding of larval ticks.

TABLE 2.

Occurrence of tick paralysis in bandicoots.

Host.	Weight gm.	Number of ticks fed.	Days on which ticks dropped from host.	Days on which host was affected.	Symptoms.	Outcome.
Band. 255	1,395	6 females	8–13	9–13	Paresis of all limbs; respiratory paralysis	Recovered
Band. 256	1,270	8 „	8	7–8	Paresis of all limbs; respiratory paralysis	Died
Band. 288*	570	1 „	13	10–14	Paretic hind-limbs	Recovered
Band. 290*	1,036	6 „	8–9	7–10	Paresis of all limbs; respiratory paralysis	Accidentally killed
Band. 292*	878	2 „	10	8–10	Paretic hind-limbs	Recovered
Band. 252	1,000	150 nymphs (approx.)	5–7	—	—	Lived
Band. 257	1,334	25 nymphs	5–7	—	—	Lived
Band. 270	939	17 „	4–7	—	—	
		8 „	5–8	—	—	Lived
Band. 293	1,000 (approx.)	485 „	5–6	4–7	Slight paresis of hind limbs	Recovered
Band. 294	1,000 (approx.)	528 „	4–5	4	Paretic hind-quarters on 4th day; dead on 5th day	Died
Band. 253	1,175	60–70 larvae	5–7	—	—	Lived
Band. 293	1,000 (approx.)	3,000 larvae (approx.)	4–5	6–7	Paresis of all limbs	Recovered
Band. 295	1,000 (approx.)	1,500 larvae (approx.)	4–5	—	—	Lived

* Canine anti-tick serum administered.

Larvae were fed upon three animals. Upon Bandicoots 253 and 295 were fed 60–70 and approximately 1,500 larvae respectively; neither animal was affected. However the engorgement of approximately 3,000 larvae on Bandicoot 293 produced paresis of all limbs on the day following the dropping of the ticks. The animal showed improvement on the following day and appeared normal on the next.

DISCUSSION.

I. holocyclus and *H. humerosa* are the two ticks commonly found on bandicoots in Southern Queensland. Although naturally infected *I. holocyclus* have not been found —few ticks have been examined—it is probable that they play an important part in the dissemination of Q fever.

In the laboratory *I. holocyclus* have been experimentally infected and found capable of transmitting infection to animals. As these ticks often attack domestic animals they provide a link between native and domestic animal reservoirs and a possible means of infection of the latter from the former. The relationship of the domestic animal reservoir to human infections is discussed elsewhere (Derrick, Smith, Brown, 1942).

I. holocyclus commonly attacks man and it may in such cases be a direct cause of infection. Q fever patients from the country occasionally give a history of tick-bite. Patient 88, who had previously lived in the city for six years, began work on a forest reserve clearing undergrowth. He developed Q fever 19 days later. He states that during that time he had three tick-bites. Patient 152, another forestry worker, said that ticks abounded after the rains began a month before his illness.

Owing to wartime emergency the weight of experimental evidence presented in this paper is not as great as would normally be sought. Nevertheless it is felt that the conclusions are legitimately drawn.

SUMMARY.

Ixodes holocylus Neumann has been found to be a potential vector of Q fever.

Larval, nymphal and adult ticks were infected experimentally with Q fever by feeding on infected laboratory animals.

The virus was passed from larvae to nymphs and from nymphs to adults but not to their progeny.

An infected adult tick infected a bandicoot upon which it fed.

In infected ticks rickettsiae were seen only in the lumen of the gut and the cytoplasm of its epithelial cells.

Although none of a small series of ticks collected from bandicoots was found to be naturally infected, it is considered that the tick may spread infection among native animals; transfer it to domestic animals from which man can become infected; and directly infect man whom it readily attacks.

Bandicoots from tick-free areas were found to be susceptible to tick-paralysis.

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STUDIES IN THE METABOLISM OF APPLES

1. PRELIMINARY INVESTIGATIONS ON INTERNAL GAS COMPOSITION AND ITS RELATION TO CHANGES IN STORED GRANNY SMITH APPLES.

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INTRODUCTION.

Metabolic activity in fruits has usually been investigated by following changes in the rate of carbon dioxide production, but recent work by Wardlaw and Leonard (1936) emphasized the desirability of investigating also the composition of the internal atmospheres of fruits. It was felt that this approach to the problem of investigating changes in ripening fruits would yield valuable information when applied to deciduous fruits, particularly stored apples. Thin protective films, mainly of wax, have been used in America to restrict moisture loss and retard ripening of certain fruits and vegetables, Magness and Diehl (1924), Smock (1936), Brooks (1937), Claypool (1939), and Platenius (1939). The use of protective coatings for prolonging the storage life of Australian fruits is being investigated in detail.

This paper is the first of a series which will deal with the physiological aspects of the work and describes the technique used and certain preliminary results. A very brief account of the methods and some of the results has been published (Trout *et al*, 1941).

Although investigations have been conducted with various fruits, the Granny Smith apple has been used for the fundamental physiological work. This variety is of major importance in Australia. Being green in colour and of good keeping quality, it is a particularly suitable variety for experimental work of this nature. Individual apples showed considerable variation in respiration rate, composition of the internal atmosphere and rate of colouring. Preliminary observations were carried out on ten apples of comparable maturity, but as the mean values for respiration rate and composition of the internal atmosphere for five fruits did not differ significantly from those for ten fruits, a unit of five apples was used in most experiments. The adequacy of a sample of five fruits was checked at intervals during the course of the investigations.

TECHNIQUE.

(a) *The measurement of respiratory activity.* The rate of carbon dioxide production by the whole fruit was determined by enclosing the fruit in a glass container and drawing over it a stream of air freed from carbon dioxide by passage over soda lime. The respired carbon dioxide was absorbed in a solution of barium hydroxide which was then titrated with hydrochloric acid. The amount of carbon dioxide liberated in a given time was obtained by this method and the results are expressed in mg. of carbon dioxide per 10 kg. of fresh weight per hour.

(b) *The determination of internal gas composition.* The method of Magness (1920) for withdrawing samples of gas by subjecting pieces of tissue to reduced pressure was tried, but was found to be unsatisfactory, as dissolved carbon dioxide was removed from solution in the cell liquid. Another difficulty in this method was that the sample was contaminated by bubbles of air which adhered to the surface of the tissue when it was immersed in mercury for the extraction. The method of Claypool (1939) in which small pieces of tissue are treated with an alkaline solution which is then titrated with acid is not satisfactory because it involves destruction of the fruit and it can be used only to determine the total amount of carbon dioxide in the fruit.

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Wardlaw and Leonard (1939) described a method by which small samples of gas can be withdrawn at intervals from the same fruit throughout the course of ripening, and their method was modified to suit the apple. A small area of the skin was sterilized by rubbing with alcohol and flaming. A sterilized cork borer was inserted into the central cavity; a small plug of tissue was removed, and a sampling tube of 10 ml. capacity inserted. The side of the fruit was used in preference to the calyx end, where a more rigid seal could be made, as the calyx was more difficult to sterilize. All apparatus was sterilized before use and precautions were taken to reduce contamination by air-borne spores. In a few cases fruit had to be discarded because of fungal infection. The sampling tube was sealed to the surface of the fruit with a small rubber washer cemented to the skin by a low melting point wax. The sampling tubes were kept in the fruit for periods up to three months and there was no injury from inserting the tubes, except to a few layers of cells in the immediate vicinity of the cut made by the cork borer.

The principle of this method is that the atmosphere in the sampling tube comes into equilibrium with the internal atmosphere of the fruit. The sampling tube was connected to a Haldane's gas analysis apparatus and a 2 ml. sample of gas extracted and analysed. The apparatus contained approximately 7 ml. of nitrogen obtained by removing oxygen and carbon dioxide from a certain volume of air. This volume of nitrogen was shunted into the caustic potash pipette and kept there while the sample of gas from the sampling tube was being taken in; the 9 ml. sample was then analysed in the usual way. In other cases the external production of carbon dioxide and the composition of the internal atmosphere were determined concurrently and the fruits were placed in glass containers used for respiration experiments with additional outlets for the sampling tubes.

Unless otherwise stated all experiments were conducted at constant temperatures.

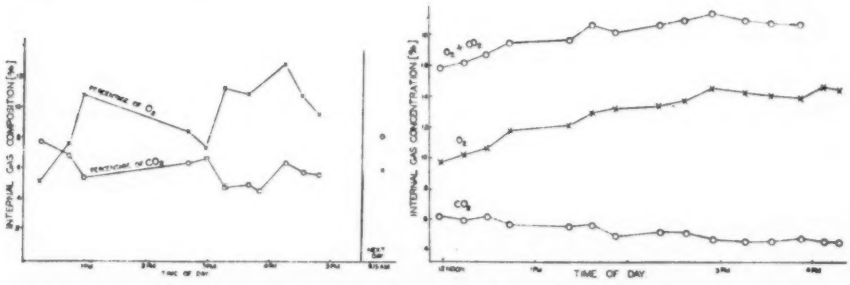
(c) *The effect of sampling.* Wardlaw and Leonard (1939) have pointed out that certain limitations are inherent in this method of sampling. A certain amount of wounding is unavoidable and the insertion of the sampling tube must cause an alteration in the composition of the internal atmosphere. They found that these effects are temporary and that normal metabolism is resumed after one or two days. These authors have also found that the manner in which the sampling tube is inserted and its position are of considerable importance in the banana because ripening takes place regionally. Their experimental evidence suggested that "the sample withdrawn is restricted to a relatively small region in proximity to the end of the sampling tube. Uniformity in the position of the sampling tube is therefore essential where close comparisons are being made between fruits". In our experiments the sampling tube was inserted in the central cavity of the fruit and comparisons between fruits are based on measurements of the composition of the atmosphere in this region. In the Granny Smith apple there is normally no connection through the calyx between the central cavity and the outside air. Nevertheless the possibility of leakage at this point was investigated. It was found that the composition of the gas in the central cavity was altered to the same extent by sealing the calyx as by sealing other parts of the skin of the same surface area. Experiments in which sampling tubes were inserted at different levels in the one apple showed that, for the fruits used in these experiments, there was practically no gradient in gas composition across the flesh.

A possible objection to the technique is the effect of repeated sampling on the internal gas composition of the fruit. The sampling bulb considerably increases the volume of gas available to the central cavity and puts the fruit under artificial conditions of aeration immediately after the sampling tube is inserted, but should not affect the fruit markedly after the initial period of equilibration. The continuous withdrawal of samples from the sampling tube will increase the internal oxygen concentration and decrease the internal carbon dioxide concentration in a way that does not occur under natural conditions. Thus, every time a sample is withdrawn from the fruit, the pressure in the sampling bulb and central cavity will be reduced until the gas removed is replaced by air entering through the skin of the fruit or by gas withdrawn from solution in the tissue. If the reduction in pressure is small, there should be no serious error in the composition of the sample withdrawn,, due to dragging carbon dioxide from solution. Further, if the subsequent intake of air into the fruit is not rapid, there should be no serious error due to air drawn into the sample.

Several experiments were conducted to determine the effect of reducing the pneumatic pressure during sampling. In these experiments two holes were bored into the centre of the same apple, and a sampling tube with a volume of 10 ml. was fitted into one and a 3-way tap and manometer into the other. The manometer and the sampling tube were both open to the central cavity when no samples were being taken. While a sample was being taken the stopcock to the manometer was closed. Immediately after the sample was withdrawn the stopcock was opened so that the sampling tube and manometer were again both open to the central cavity. Assuming that the fruit has a volume of 120 ml. and the sampling tube a volume of 10 ml., the reduction in pressure in the air system (assuming the intercellular space system equivalent to $\frac{1}{3}$ of the volume of the fruit), will be equal to about 0·04 atmospheres, or 46 cm. of medicinal paraffin. The greatest reduction of pressure observed was 13·5 cm. which indicated that 71 p.c. of the readjustment of the gas pressure took place almost immediately. Subsequently the reduction of pressure equivalent to the 13·5 cm. of paraffin came back to normal in a period of about five

minutes. It is thus unlikely that the initial rapid equilibration of pressure is confined to the readjustment of the gas within the fruit, and it appears as though some air is drawn rapidly in through the skin. This effect of sampling has been mentioned by Wardlaw and Leonard (1939) but was thought to be worthy of further investigation. In some of the later experiments the sampling tubes were clipped off from the apple before samples were taken and the results were the same as when they were not clipped off. This shows that the sample was not contaminated by air drawn through the fruit.

As a result of sampling the fruit is irrigated with a small quantity of outside air drawn in every time a sample is taken. The effect of the repeated withdrawal of samples upon the composition of the gas within the fruit is shown in several experiments in which samples were taken at short intervals throughout one day. In the first experiments (Fig. 1) sampling at short intervals caused a rise in oxygen concentration in the centre of the fruit from 5·1 p.c. to 10·8 p.c. and a fall in carbon dioxide from 7·7 p.c. to 5·4 p.c. When the fruit was left for a period of one hour 40 minutes before the next sample was taken a reversion towards the initial values was observed. Further sampling showed a general tendency to increase the oxygen concentration and to decrease the carbon dioxide concentration, though the two curves are rather irregular After a lapse of sixteen hours the oxygen concentration differed from the previous day's initial value by less than 1 p.c. and the carbon dioxide by less than 0·5 p.c.



Figs. 1, 2. Graphs showing the internal concentrations of oxygen and carbon dioxide observed during two experiments in which the internal atmosphere was sampled at short intervals over a period of several hours. The experiments were carried out at 21·1° C.

The effect of repeated sampling over short periods was shown in two other experiments (one of which is illustrated in Fig. 2), in which the carbon dioxide showed a steady fall and the oxygen rose. The oxygen concentration (Fig. 2) rose from 9·7 p.c. to a maximum value of 14·7 p.c., while the carbon dioxide fell only from 6·2 p.c. to a minimum of 4·8 p.c. In the second experiment the oxygen rose from 7·0 p.c. to a maximum of 16·0 p.c. while the carbon dioxide fell from 6·6 p.c. to 4·4 p.c. during the same period. The greater change in the percentage of oxygen than in that of carbon dioxide would be expected because the external concentration of oxygen is 20·9 p.c. compared with an internal concentration of 7·0 p.c. (in the second case), whereas the external concentration of carbon dioxide is very small compared with an internal concentration of 6·6 p.c. Moreover, the concentration of carbon dioxide in the gas phase will be increased by carbon dioxide coming out of solution to a new equilibrium. Also the greater oxygen supply may tend to increase the respiration rate, which would increase the concentration of carbon dioxide in the internal atmosphere.

The experiment (Fig. 1) in which a recovery of oxygen and carbon dioxide concentrations was shown after sixteen hours indicated that, in sampling once every 24 hours, ample time is provided for the restoration of equilibrium in composition. This is borne out by numerous results in which the concentration obtained from day to day showed no variation which could be attributed to the effect of withdrawing the sample.

(d) *The resistance of the fruit to the diffusion of carbon dioxide and oxygen.*

Kidd and West (1934) and Wardlaw and Leonard (1936) have realized that consideration of the permeability of fruits to gaseous diffusion is very important in studies of this kind. Kidd and West obtained a rough measure of the "porosity" of their apples by dividing the value for carbon dioxide production by the corresponding value for the difference between the internal and external concentrations of carbon dioxide.

The following methods have been suggested by E. W. Hicks, of the Division of Food Preservation, for the quantitative expression of the resistance of the fruit to the passage of carbon dioxide and oxygen.

Assuming that the resistance of the flesh is very small in comparison with that of the skin (see later experiments), let v_c, v_o, v_n be equivalent volumes of fruit per sq. cm. surface for carbon dioxide, oxygen and nitrogen respectively then

$$v_c = v_a + \alpha_c v_w$$
$$v_o = v_a + \alpha_o v_w$$
$$v_n = v_a + \alpha_n v_w$$

where v_a is the volume of intercellular space per sq. cm. surface; v_w is the tissue volume per sq. cm. surface, and α_c, α_o, α_n the volumetric solubility coefficients in the tissue of carbon dioxide, oxygen and nitrogen respectively.

Let C_c, C_o, C_n be the volumetric concentrations of the three gases in the intercellular spaces. The corresponding external concentrations are 0·21 for oxygen and 0·79 for nitrogen.

Define resistance coefficients r_c, r_o, r_n by $Q = \frac{\delta C}{r}$ where Q is the volume of gas transferred per sq. cm. per sec. and δC the concentration difference between inside and out.

The internal pressures vary very little from atmospheric so that unless $\frac{\delta C_c}{r_c} + \frac{\delta C_o}{r_o} + \frac{\delta C_n}{r_n} = 0$ there must be mass movement either in or out to maintain equilibrium.

Let the rate of air inflow be x c.c. per sq. cm. per sec.

Assume R c.c. of carbon dioxide produced per sq. cm. of surface per sec. and qR c.c. the corresponding amount of oxygen absorbed.

Then for carbon dioxide $\frac{dC_c}{dt} = \frac{1}{v_c}\left(R - \frac{C_c}{r_c}\right)$

for oxygen $\frac{dC_o}{dt} = \frac{1}{v_o}\left(-qR - \frac{C_o - 0{\cdot}21}{r_o} + 0{\cdot}21\,x\right)$

$$= \frac{1}{v_o}\left(-qR + \frac{\delta O}{r_o} + 0{\cdot}21\,x\right)$$

and for nitrogen $\frac{dC_n}{dt} = \frac{1}{v_n}\left(-\frac{C_n - 0{\cdot}79}{r_n} + 0{\cdot}79\,x\right)$

$$= \frac{1}{v_n}\left(0{\cdot}79\,x - \frac{\delta N}{r_n}\right)$$

For a steady state $\frac{dC_c}{dt} = \frac{dC_o}{dt} = \frac{dC_n}{dt} = 0$, i.e. $R - \frac{C_c}{r_c} = 0$, $-qR + \frac{\delta O}{r_o} + 0{\cdot}21\,x = 0$, and $0{\cdot}79\,x - \frac{\delta N}{r_n} = 0$. Therefore $r_c = \frac{C_c}{R}$, $r_o = \frac{\delta O}{qR - 0{\cdot}21\,x}$ and $r_n = \frac{\delta N}{0{\cdot}79\,x}$.

These equations will be almost exact if $\frac{dc}{dt}$ is small compared with the other quantities involved.

In these equations C_c, δO, δN and R are all known, and q could also be determined. But it would be impossible to determine x without further information.

The resistance to carbon dioxide is obtained in arbitrary units by multiplying the percentage internal concentration of carbon dioxide by 100 and dividing the product by the respiration rate in mg. per 10 kg. of fresh weight per hour.

Because of the importance of r_o an approximate equation has been derived. If the diffusion paths were all gaseous it could be assumed, without serious error, that $r_n = r_o$.

$$\text{Thus } r_o = \frac{\delta O + 0{\cdot}27\,\delta N}{qR}.$$

In water, however, the solubility of nitrogen is approximately half that of oxygen so that it can be assumed that $r_n = 2r_o$ (approx.).

$$\text{Thus } r_o = \frac{\delta O + 0{\cdot}13\,\delta N}{qR}.$$

Since the second term in the numerator of the right hand side of these two equations is considerably smaller than the first term the use of a mean value, viz.,

$$r_o = \frac{\delta O + 0{\cdot}2\,\delta N}{qR}.$$

seems reasonable as an approximation. It is unlikely that $\frac{r_n}{r_o}$ will be exactly 2·0 so that the values calculated using the approximate equation should be used with some caution.

In this equation δO and δN are expressed as percentage concentrations and R is the respiration rate in mg. of carbon dioxide per 10 kg. of fresh weight per hour. The resistance to oxygen was obtained in arbitrary units by multiplying this value by 100.

In our experiments the respiratory quotients were not determined. It was assumed that respiration was of the normal type and that the respiratory quotient was about one. Alcohol estimations since carried out by F. E. Huelin of the Division of Food Preservation, showed that there was no appreciable increase in the alcohol content of Granny Smith apples during storage at 1·1° C. or 18·3° C. until very late in storage life when the apples were showing signs of breakdown. These data support the assumption that the respiratory quotient was constant during the course of the experiments, but it must be remembered that anaerobic respiration is not always accompanied by alcohol formation. In future experiments the exact value of the respiratory quotient should be determined.

Evidence that most of the resistance is in the skin is provided by the following experiments. Three apples were taken from store late in the season when the internal oxygen concentration was low. When the whole of the skin was removed the internal oxygen concentration increased from 6 p.c. to 20 p.c. and the carbon dioxide fell from 7·2 p.c. to 2·4 p.c. When a piece of skin about 2 cm. in diameter was removed, the internal carbon dioxide concentration decreased, and the internal oxygen concentration increased (see Table 1). The fact that the internal atmosphere still contained a relatively high percentage of carbon dioxide after skin removal may have been due to some resistance resulting from the drying out of the exposed cells of the flesh.

TABLE 1.

The effect of removing a small portion of the skin on the composition of the internal atmosphere of Granny Smith apples removed from cool storage on 16/10/40.

	Apple A.		Apple B.		Apple C.	
	Int. CO_2 (%).	Int. O_2 (%).	Int. CO_2 (%).	Int. O_2 (%).	Int. CO_2 (%).	Int. O_2 (%).
Before treatment	5·5	7·1	5·2	9·6	4·4	9·4
After treatment	3·5	15·2	3·5	14·2	2·9	16·7

TABLE 2.

The effect of puncturing and removing portions of the skin on the composition of the internal atmosphere of Granny Smith apples removed from cool storage on 2/9/41.

		Before treatment.		24 hours after treatment.	
Treatment.	Fruit.	Int. CO_2 %.	Int. O_2 %.	Int. CO_2 %.	Int. O_2 %.
Three pin pricks	a	5·9	8·1	5·8	9·9
	b	6·3	4·6	6·4	7·6
Twelve pin pricks	a	5·9	10·1	4·9	13·1
	b	4·6	13·0	4·0	14·5
One area one sq. cm. removed	a	6·0	6·9	5·6	9·0
	b	5·6	4·6	5·0	10·6
Four areas each one sq. cm. removed	a	6·4	7·8	3·3	16·8
	b	6·0	11·5	3·6	16·9
Peeled	a	6·0	10·6	1·4	19·1
	b	6·5	6·9	1·0	19·7

In 1941 the experiment was repeated, pieces of skin of various sizes being removed. When the whole of the skin was removed the composition of the internal atmosphere 24 hours after treatment was very close to that of air. The results are given in Table 2.

It has already been mentioned that when sampling tubes were inserted to different depths in the one fruit, the gas composition throughout the flesh appeared to be uniform. This further supports the hypothesis that resistance is mainly in the skin.

THE RELATION BETWEEN INTERNAL GAS COMPOSITION AND EXTERNAL CARBON DIOXIDE PRODUCTION.

Wardlaw (1940) found that the respiration rate, internal gas composition and carbon dioxide content of bananas was related to the composition of the atmosphere by which the fruit was surrounded. Parija (1928) showed that low external concentrations of oxygen depressed the respiration of Bramley's Seedling apples. Kidd and West (1920) working with Bramley's Seedling apples found that an external concentration of 5 p.c. oxygen not only delayed the onset of the climacteric but also considerably reduced its magnitude. A marked depression in the respiratory activity of post-climacteric Sturmer Pippin apples was produced by a 5 p.c. concentration of carbon dioxide but no such marked effect was produced on apples in the preclimacteric phase, although the onset of the climacteric was considerably delayed.

Smock (1936) treated several varieties of apples and pears with various waxy skin coatings and in most cases ripening was delayed. He found that waxing decreased the respiration rate and suggested that this may have been due to a reduced supply of oxygen to the fruit or to an increased concentration of carbon dioxide within the fruit itself. Using the method of Magness (1920) he found an increased concentration of carbon dioxide in waxed pears. Brooks (1937) increased the storage life of tomatoes by waxing which increased the percentage of carbon dioxide in the internal atmosphere Platenius (1939) considerably reduced the shrinkage of various vegetables by waxing treatments and showed that a marked decrease in the respiration rate of carrots as a result of waxing was associated with a decrease in oxygen consumption.

In July, 1940, Granny Smith apples were treated with various skin coatings and stored at 21·1° C. The fruit was enclosed continuously in respiration containers and might therefore have differed in behaviour from similar fruits stored in the usual way (see next Section). However the results shown in Table 3 are of value in correlating external carbon dioxide production with internal oxygen concentration.

TABLE 3.

Respiration rate and internal atmosphere of Granny Smith apples treated with various skin coatings.

	No. of replicate.	Internal CO_2 (%).	Internal O_2 (%).	Respiration rate.*
Controls	1	4·5	12·4	138
	2	3·7	16·4	156
	3	3·2	11·6	119
Coating A	1	5·1	8·8	132
	2	4·5	7·5	97
	3	5·1	8·2	92
Coating B	1	3·0	11·1	140
	2	3·8	7·8	117
	3	3·5	7·5	95
Coating C	1	4·8	6·0	115
	2	4·1	5·0	88
Coating D	1	4·4	4·5	93
	2	4·4	13·2	127
	3	2·8	2·8	74
Coating E	1	3·7	5·0	92
	2	5·1	5·1	76
Coating F	1	2·7	8·2	109
	2	2·8	9·0	120
	3	4·0	4·2	91
Coating G	1	3·3	7·3	97
	2	2·9	10·2	113

* Mg. per 10 kg. per hour.

The respiration rates of treated fruits were less than those of control fruits. The respiration rates of both control and treated fruits were either steady or gradually decreased with time, no climateric rise being observed.

The treatments greatly reduced the internal oxygen concentration, the mean value for treated apples being 7·3 p.c. and for untreated fruits 13·4 p.c. The data for both treated and control fruits have been used to determine the relation between composition of the internal atmosphere and the external respiration rate. The regression coefficient of respiration against internal oxygen has been calculated, using the mean values of readings on individual apples taken throughout the course of the experiment. This regression coefficient was 5·674 and was highly significant ($P < 0·001$).

In some of the 1940 experiments high internal oxygen concentrations were associated with high respiration rates within a sample of untreated fruits. For one such fruit the regression coefficient was 18·742 and was highly significant ($P << 0·001$). Results for 1941 (to be published later) indicate that this correlation held for fruits of commercial maturity picked in mid-April and taken from store during the first half of the year. In 1941 experiments were conducted to determine the relationship between internal oxygen concentrations from 1–18 p.c., and the respiration rate. A correlation between the two was obtained for concentrations of oxygen above approximately 5 p.c. Kidd, West and Kidd (1927) have given a set of diagrams depicting the probable relationship between the rate of respiration and the concentration of oxygen in the external atmosphere at 1·1° C. and these show a slight deviation from a linear relationship over the range 5–21 p.c. oxygen. The same workers have given diagrams showing a probable linear relationship between external carbon dioxide concentration and respiration rate.

In untreated Granny Smith apples the internal carbon dioxide did not always show marked differences with different respiration rates. However, the expected changes would be numerically small compared with those in oxygen values and may have been masked by experimental errors and natural fluctuations. Another possibility is that differences in resistance to gaseous exchange were responsible for the lack of correlation between respiration rate and internal carbon dioxide concentration. In the experiment of July, 1940, there was no significant difference between the internal concentrations of carbon dioxide in coated and control fruits two weeks after treatment. There was no correlation between internal carbon dioxide concentrations and external respiration rate.

It is thus evident that a reduction in the internal oxygen concentration is not necessarily accompanied by an increase in internal carbon dioxide. It can therefore be concluded that, in these experiments, the reduced respiration rate is probably due to the fall in the internal oxygen concentration.

CHANGES IN RESPIRATION AND INTERNAL GAS COMPOSITION IN TIME.

Five Granny Smith apples, picked on 12/4/40, were taken from store at 0·0° C. on 10/6/40 and sampling tubes were inserted. The fruit was kept at 21·1° C. and the composition of the internal atmosphere was determined every 24 hours. The apples were green and hard at the beginning of the experiment. After 37 days, when the experiment was concluded, the apples were fully yellow and wrinkled. The initial internal oxygen concentrations ranged from 15·5 p.c. to 19·0 p.c. while the corresponding carbon dioxide concentrations were from 6·7 p.c. to 3·2 p.c. The sum of the internal oxygen and carbon dioxide concentrations was approximately 22 p.c., slightly higher than the corresponding total for the surrounding air. For fifteen days the internal oxygen concentration remained at a high level. It then fell until the end of the experiment when the average value was 5·6 p.c. The rate of fall varied in individual fruits. Fluctuations were observed in the downward trend in oxygen concentration; these were too great to be due to experimental errors. Similar fluctuations have been observed frequently in the respiratory drift of other fruits and so far their cause has not been investigated. The mean internal carbon dioxide concentration showed no change during one experiment and was approximately 6 p.c. A typical result is shown in Fig. 3. In one fruit a significant increase in carbon dioxide concentration took place (Fig. 4).

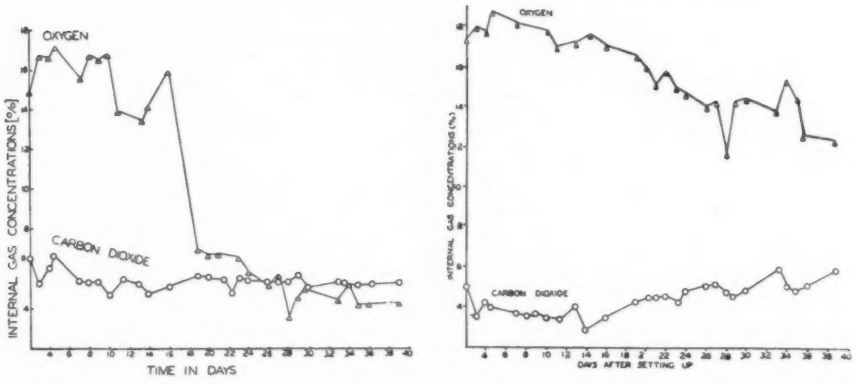
The general decrease in oxygen concentration could be due to two causes acting either together or independently.

(1) Increased rate of utilization of oxygen due to increased respiration rate.

(2) Increased resistance of the fruit to gaseous diffusion.

To investigate these possibilities an attempt was made to determine the relationship between changes with time in the internal gas composition of the fruit and the external carbon dioxide production. Five fruits were fitted with sampling tubes and set up in bottles through which a stream of air freed of carbon dioxide was passed. The fruits were similar to those used previously and the experiment was started on 18/6/40. During the course of the experiment three fruits developed mould, and consequently later results are based on measurements from two fruits only.

The relation between the external carbon dioxide production and the composition of the internal atmosphere for one of these two fruits is shown in Fig 5. Over a period of five weeks there was little change in the internal oxygen concentration which remained at a high value. The rate of external production of carbon dioxide was high initially and fell rapidly for about fourteen days and thereafter remained relatively steady. The internal concentration of carbon dioxide was relatively high initially, fell slowly for about fourteen days and thereafter remained approximately steady. Thus, in this experiment, changes in the rate of external production of carbon dioxide were not associated with changes in the internal concentrations of oxygen but the small changes in internal carbon dioxide concentration were in the same direction as the changes in the rate of external production of carbon dioxide.



Figs. 3, 4. Graphs showing the trends of internal oxygen and carbon dioxide concentration in each of two fruits at 21·1° C.

The remarkable feature about this experiment was that the internal oxygen concentration of these fruits in bottles remained high for 38 days whereas freely exposed fruits used in the earlier experiment showed a marked fall in oxygen concentration after fifteen days. In the first experiment the fruit took five weeks to reach full yellow colour while in this experiment the fruit in bottles took fifteen weeks to reach the same condition. The two lots of apples could be regarded as being of similar age as they were picked on the same day and kept in cool storage for about the same time. A striking difference in behaviour between fruits kept in bottles for determining carbon dioxide output and similar fruits kept in cases frequently has been observed. Owing to the fact that the behaviour of single fruits enclosed continuously in respiration containers does not usually parallel that of fruits stored in the usual way this difference in behaviour is being investigated further. It has been necessary, therefore, to devise a technique for measuring the composition of the internal atmosphere and the rate of carbon dioxide production concurrently without keeping the fruits in the containers all the time. This was done by making the sampling tubes detachable and the fruits were enclosed in the respiration containers only over the periods during which the respiration was being measured. At the end of a run on respiration the pipettes were replaced and a period of at least 40 hours elapsed before a sample of the internal atmosphere was taken.

This technique has been applied in all subsequent experiments and was used on 29/10/40 to determine the relationship between the composition of the internal atmosphere and external respiration rate. The results are given in Fig. 6. Until the 24th day the fall in the rate of external carbon dioxide production was accompanied by a fall in internal oxygen concentration. The decrease in internal oxygen concentration accompanied by a decrease in respiration rate indicates that there was some increase in the resistance of the fruit to oxygen diffusion.

The increase in the resistance of the fruit to gaseous diffusion with increasing age is shown clearly in the following table of results obtained with apples observed at 21·1° C. after various periods of storage at 1·1° C.

Thus, in the sample removed on July 13th, the fall in respiration rate with time is associated with a fall in internal oxygen concentration. The fall in oxygen concentration is probably due to an increase in the resistance of the fruit to gaseous diffusion. In the earlier sample (June 13th) the fall in respiration rate with time

TABLE 4.

The effect of time on the resistance of Granny Smith apples to gaseous diffusion.

(A) *Removed from cool storage at 1·1° C. on June 13.*

Days at 21·1° C.	External Respiration.	Int. CO_2 %.	Int. O_2 %.	R_c	R_o
5	265	1·8	19·0	0·6	0·6
13	190	1·9	19·0	0·4	0·4
18	175	1·5	19·3	0·5	0·5
20	145	2·1	18·6	1·2	1·2
26	140	1·6	19·2	1·1	1·2

(B) *Removed from cool storage at 1·1° C. on July 13.*

Days at 21·1° C.	External Respiration.	Int. CO_2 %.	Int. O_2 %.	R_c	R_o
8	164	3·2	17·6	1·9	1·9
16	163	3·9	16·9	2·4	2·4
18	159	3·6	18·2	2·3	2·3
22	156	3·9	17·2	2·5	2·5
25	156	3·8	12·4	2·4	4·8

(C) *Removed from cool storage at 1·1° C. on October 29.*

Days at 21·1° C.	External Respiration.	Int. CO_2 %.	Int. O_2 %.	R_c	R_o
4	280	8·4	5·2	3·0	5·0
8	160	6·4	4·2	4·0	9·1
12	130	5·5	5·0	4·2	10·5
14	145	5·2	5·0	3·6	9·0

is not associated with a fall in internal oxygen concentration. The resistance to oxygen increases with time but is still comparatively small after 26 days at 21·1° C. In the latest sample (October 29th) the fall in respiration rate with time is not associated with a fall in internal oxygen concentration but with a fall in internal carbon dioxide concentration. In this sample the initial resistance to oxygen was very high and increased further with time. It seems that in the early stages when the oxygen resistance is low, the respiration rate is not limited by internal oxygen concentration; later, as the resistance to oxygen diffusion increases, the respiration rate becomes dependent on the internal oxygen concentration; finally when the

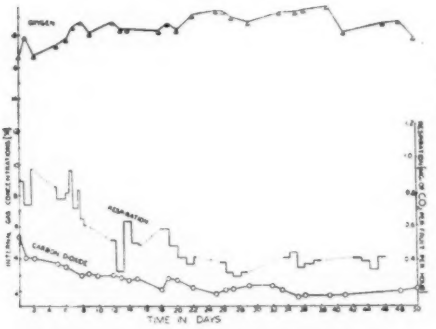


Fig. 5. Graph showing the trends of internal oxygen and carbon dioxide concentrations and respiration rate of a single fruit at 21·1° C.

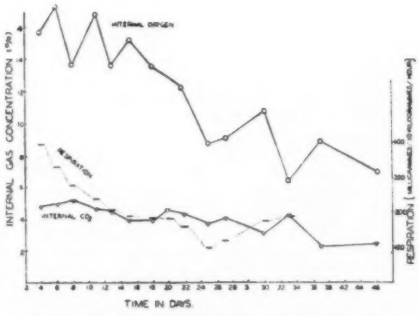


Fig. 6. Graph showing the trends of internal oxygen and carbon dioxide concentrations and respiration rate of a single fruit at 21·1° C. This fruit was taken from cold store several months later than the fruit referred to in Fig. 5.

resistance is very high on removal from store, the respiration rate is again independent of internal oxygen concentration, probably due to a change to anaerobic respiration. These conclusions are supported by later work.

THE EFFECT OF TEMPERATURE ON EXTERNAL RESPIRATION AND COMPOSITION OF THE INTERNAL ATMOSPHERE.

Gore (1911) determined the rate of carbon dioxide production of a number of fruits at different temperatures and found that the temperature coefficient of respiration varied from 2·04 to 3·20. Kidd and West (1930a) found that the Q_{10} for Bramley's Seedling apples was 3·27. In 1941 Granny Smith apples picked on April 1 (two weeks before commercial maturity) from thinned trees, and removed from storage at 1·1° C. on 13th October had at first a Q_{10} of approximately 3·1 from 4·5° C. to 18·3° C. but after three weeks the Q_{10} was about 2·2 over the same range of temperature.

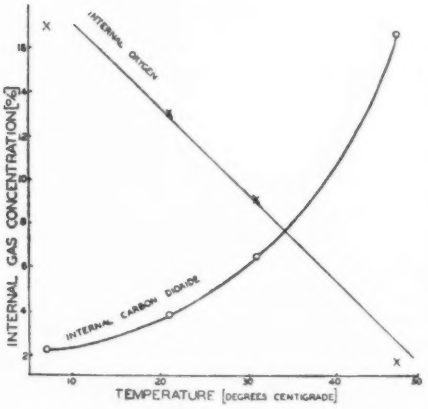


Fig. 7. The effect of increasing temperature on the internal concentrations of oxygen and carbon dioxide.

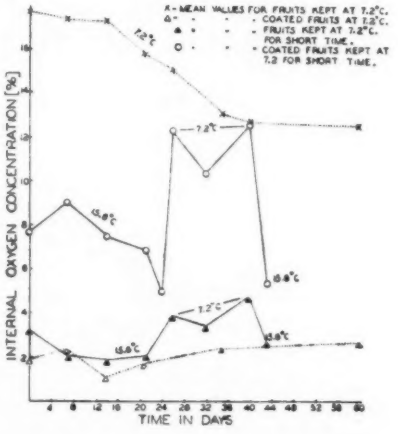

X - MEAN VALUES FOR FRUITS KEPT AT 7.2°C.
COATED FRUITS AT 7.2°C.
FRUITS KEPT AT 7.2°C. FOR SHORT TIME.
COATED FRUITS KEPT AT 7.2 FOR SHORT TIME.
INTERNAL OXYGEN CONCENTRATION [%]
TIME IN DAYS

Fig. 8. Graph showing the effects of lowering and raising the temperature on the mean internal oxygen concentration of coated and control fruits.

Various workers have investigated the effect of temperature on the composition of the internal atmosphere. Magness (1920) found that the internal carbon dioxide concentration increased and the internal oxygen concentration decreased with increasing temperature. At lower temperatures the sum of the percentages of oxygen and carbon dioxide was equal to 21 p.c., the value for air, but at higher temperatures this total gradually increased. It was suggested that this was probably due to anaerobic respiration which would cause an increase in the concentration of carbon dioxide without a corresponding decrease in oxygen. Kidd and West (1923) presented data obtained at Cambridge by T. Ekambaram on Bramley's Seedling apples and the results were similar to those reported by Magness. The composition of the internal atmosphere of Granny Smith apples was determined over the range of temperatures 7° C. to 47° C. and the results are shown in Fig. 7. Readings were not taken during the first few days at each temperature to avoid the temperature transition effect. As the temperature increased, the internal concentration of carbon dioxide also increased, and the concentration of oxygen decreased. At each temperature the total of the two gases was always less than 21 p.c. These effects are no doubt related to the rise in respiration rate due to increased temperature. In an experiment commenced on 23/10/40 it was found that on transferring fruits from 15·6° C. to 7·2° C. there was a marked rise in internal oxygen concentration and a fall in internal carbon dioxide (Fig. 8). When the fruit was again placed at 15·6° C. the original values were regained.

Numerous experiments have since been carried out to determine the effects of temperature on the respiration rate and the composition of the internal atmosphere, and these results will be published later.

THE EFFECT OF THE COMPOSITION OF THE EXTERNAL ATMOSPHERE ON THE COMPOSITION OF THE INTERNAL ATMOSPHERE.

Wardlaw (1936) found that there was a close relation between the composition of the internal atmosphere of bananas and pawpaws and that of the atmosphere in which the fruit was stored. The effect of the external atmosphere on the internal gas composition of apples was determined in 1940 at 21·1° C. and 7·2° C. The fruits were fitted with sampling tubes and placed in sealed desiccators. The atmospheres in the desiccators were controlled by ventilation. The pipettes protruded from the desiccators so that the internal gas concentrations of the fruit could be measured without disturbing the external atmospheres. Changes in external atmosphere were reflected in the internal atmosphere. The effects at the lower temperature are shown in Fig. 9 left graph.

In another experiment the effect of low external oxygen concentrations in the absence of external carbon dioxide was investigated by keeping the fruit in desiccators in which soda lime had been placed to absorb carbon dioxide. The internal oxygen concentration closely followed changes in the external atmosphere. (Fig. 9 right graph.)

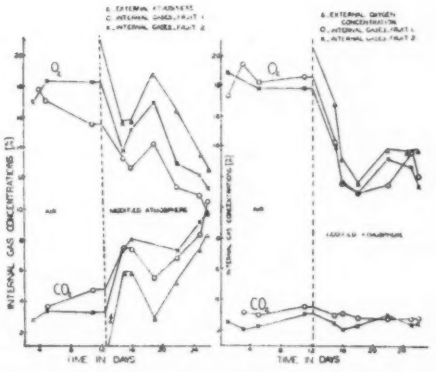


Fig. 9. The effect of modified external atmospheres on the internal atmospheres at 7·2° C. The left graph shows effects of external atmospheres containing carbon dioxide; the right graph shows effects of external atmospheres containing no carbon dioxide.

THE RELATION BETWEEN INTERNAL OXYGEN CONCENTRATION AND THE RATE OF COLOURING.

Smock (1939) found that waxing certain varieties of pears and Yellow Newtown apples retarded their subsequent colouring. Kidd and West (1930b) have reported that at 10° C. a concentration of 15 p.c. carbon dioxide in the external atmosphere doubled the time taken by Bramley's Seedling apples to reach a given stage of colour and that the percentage of oxygen with which the carbon dioxide was mixed did not influence the result. The effect of concentrations of oxygen alone was as pronounced as that of carbon dioxide, and in 5 p.c. oxygen the fruit took twice as long to yellow as in air. These authors (1931a) have also found that decreased concentrations of oxygen down to 5 p.c. had little effect upon the rate of carbon dioxide production of bananas but retarded colour development.

Kidd and West (1933b) have also found that the climacteric occurred earlier in 50 p.c. oxygen than in air, but in 5 p.c. oxygen the onset of the climacteric was definitely delayed. When apples were kept in an atmosphere of nitrogen no climacteric occurred even after treatment with ethylene. When Winter Nelis pears (Kidd and West, 1931b) were kept indefinitely in atmospheres containing less than 5 p.c. oxygen they did not ripen. It seems therefore that the rate of colouring is affected by the concentration of oxygen in the external atmosphere, and that the development of colour is sometimes associated with metabolic processes resulting in a climacteric of respiration. In many of our experiments Granny Smith apples have developed their full yellow colour apparently without having passed through a climacteric.

In this apple the rate of colour development is correlated with the internal oxygen concentration both at 7·2° C. and 21·1° C. Colour development has been completely inhibited when the internal concentration has been reduced by treat-

ment with skin coatings to values below about 3 p.c. or when the external concentration is reduced below about 8 p.c. It has also been observed that samples of untreated apples differ in their rates of colouring after removal from cool storage and those which colour fastest generally have the highest internal oxygen concentration. It is probable that the retardation of colouring by coating is due to the reduction of internal oxygen. Very little is known about the processes involved in the colouring of fruits. The development of colour may occur without accompanying ripening changes, or fruit may ripen without colouring.

SUMMARY.

The techniques used for determining the respiration rate and the composition of the internal atmosphere of apples are described and discussed in full.

A method is suggested for the quantitative expression of the resistance of the fruit to the passage of carbon dioxide and oxygen.

The internal atmospheres of Granny Smith apples were varied artificially by the application of skin coatings. Most of the coatings used in 1940 decreased both the internal oxygen concentration and the respiration rate.

There was a highly significant correlation between internal oxygen concentration and respiration rate for coated fruits, but this did not always hold for untreated fruits. The rate of respiration was probably limited by internal oxygen supply. The internal concentration of carbon dioxide did not appear to be increased by coating and was not correlated with the respiration rate in either coated or control fruit.

Untreated Granny Smith apples were held at 21·1° C. after various periods of storage at 1·1° C. The respiration rate at 21·1° C. decreased with time to a fairly steady value. This decrease in respiration rate was frequently associated with a decrease in the concentration of internal oxygen. The decrease in internal oxygen was probably due to an increase in the resistance of the fruit to gaseous diffusion.

The effects of changes in temperature on the respiration rate and the composition of the internal atmosphere were investigated. For temperatures between 7° C. and 47° C. the internal atmosphere contains more carbon dioxide and less oxygen as the temperature rises.

Changes in the composition of the external atmosphere were reflected by changes in the internal atmosphere.

In untreated fruits the rate of colouring was related to the internal oxygen concentration. Treatments generally retarded colouring both at 7·2° C. and 21·1° C. The delay in colouring after treatment may have been due to the reduction of internal oxygen supply.

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